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TITLE: Ex Vivo Expansion of HER2-Specific T Cells for the Treatment of HER2-Overexpressing Breast Cancer

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Adoptive T cell therapy has the potential to eradicate existing malignancy in humans. I have been investigating the efficacy of adoptive T cell therapy at eradicating malignancy in the neu-transgenic mouse model of human breast cancer. Helper peptides of neu have been identified to which T helper cell lines can be generated. Cell lines derived using these peptides, were tested for the ability to eradicate existing bulky malignancy. T cell injection resulted in a partial tumor response. I have been investigating how to improve efficacy. Polyclonal T cell lines recognizing multiple epitopes are more effective. I have observed that immunosuppressive T cells associate with these breast tumors. Prior elimination of these cells may improve outcome. A monoclonal antibody therapy strategy has also been developed that will be tested in combination with adoptive T cell transfer. This is an adaptation of this murine model to better reflect current strategies in humans (i.e. Herceptin). Techniques for optimal ex vivo expansion of human HER-2/neu-specific T cells are also continuing to be developed. Antigen-specificity of cultures can be preserved following rapid expansion with anti-CD3/CD28 beads. The findings in the animal model and ex vivo expansion of human T cells will be directly translated to human clinical trials of adoptive T cell therapy.

14. SUBJECT TERMS
Adoptive immunotherapy, T cells, immunology, peptide-based vaccine, cytolytic T cell, T helper cell, cytokines

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Introduction:

A central hypothesis of this grant proposal is that tumors can be eradicated in vivo using adoptive transfer of tumor-specific T cells. However, many of the underlying principles of adoptive T cell therapy are not well understood. This grant describes experiments that aim to elucidate some of these principles. The specific aims of this proposal are to, (1) determine the immune effector arm most effective in the eradication of rat neu overexpressing tumors in neu-tg mice using adoptive immunotherapy of rat neu specific CD8+ and CD4+ T cells and (2) determine the feasibility of the expansion of HER2 specific CD4+ and CD8+ T cell lines from patients previously immunized with a HER2 peptide-based vaccine.

Body:

1. To determine the immune effector arm most effective in the eradication of rat neu-overexpressing tumors in neu-tg mice using adoptive immunotherapy. The work in year 3 of the proposal has focused further on generating T cells, characterizing the T cells *ex vivo*, and infusing them into animals to assess an anti-tumor response. In general, I have confirmed my previous findings and established new principles for adoptive T cell therapy. Mice were immunized with peptides derived from the neu protein sequence that had been shown to be immunogenic and to generate a tumor specific response. In my previous report, I had shown that a potent immune response is able to generate antigen-loss variants. To further enhance the adoptive T cell therapy model, I established a cell line from this antigen-loss tumor. After several passages, I characterized the tumor for growth in neu-tg and the parental mice, FVB/N. The *in vivo* growth of my original neu^+ MMC cell line and the new antigen-negative variant cell line, ANV, is shown in Fig. 1. MMC are unable to grow in parental FVB/N as previously reported and are able to grow in neu-tg mice where neu is a self-antigen (Fig. 1A). ANV, in contrast, lacks neu expression and is able to grow rapidly in both FVB/N and neu-tg mice (Fig. 1B). Also, Figure 1C shows that for the MMC tumor cells, neu is a biologically relevant antigen as growth of the tumor cells is inhibited by 7.16.4., an inhibitory mouse anti-neu antibody. In contrast, the growth of ANV was unaffected by inclusion of the antibody.

T cell lines generated against neu-derived CD4 helper peptides p781, p932, and p1166 were tested for activity against peptide and tumor cell lines MMC and ANV (Fig. 2). Spleens from immunized animals were removed and peptide-specific T cells expanded *ex vivo* using IL-2. Peptide-specific T cells were generated against all 3 neu peptides after 1 *in*

vitro stimulation (IVS) (Fig. 1A). Consistent with my previous studies, the peptide-specific T cells were able to recognize neu+ MMC tumor cells. Specificity of the T cells for neu peptides was confirmed by demonstrating that the cells were unable to recognize the ANV cell line which does not express neu (Fig. 1). Previously I had shown that peptide-specific T cells were able to mediate partial inhibition of tumor growth. I have begun to question how this effect can be improved. Although there are a number of factors that should be considered, one possibility is that the cells have reduced avidity compared to cells from animals that are not tolerized against neu. If this were the case, then peptide-specific T cells derived from parental FVB/N may have better tumor inhibition. I examined this by infusing peptide-specific T cells derived from parental FVB/N and compared this to cell lines generated from the tolerized neu-tg mouse as shown in Figure 3A. p1166-specific cells lines derived from neu-tg demonstrated a similar level of tumor growth inhibition as those derived from FVB/N. Another possibility for the tumor growth inhibition was not complete was the generation of antigen-negative variants. I examined this by staining fresh tumor samples with neu-specific monoclonal antibody and assessing the expression of neu at the cell surface (Fig. 3B). I observed that tumors still retained neu expression comparable to that in control animals. It is possible, however, that expression of p1166 peptide in MHC class II is diminished in the tumors. One way to circumvent this problem is by the use of polyclonal T cell lines. Polyclonal T cell lines were generated *in vitro* by repetitive restimulation using whole irradiated tumors. These T cells lines were infused into tumor bearing animals and completely inhibited tumor growth with a single infusion (Fig. 4A). These polyclonal lines were examined by ELIspot analysis testing for both IFN-γ and IL-4 against both MMC and ANV tumor cell lines (Fig. 4B). Polyclonal T cell lines contained high levels of IFN-γ and IL-4 responding T cells. Of those that produced IFN-γ, the vast majority recognized MMC, with a smaller fraction (12%) recognizing ANV. These results suggest that the major IFN-γ response is directed against neu. A large fraction of the cells also secreted IL-4 targeting both MMC and ANV. Although the importance of Th1 type IFN-γ-secreting cells for anti-tumor immunity is well established, the role of IL-4-producing Th2 cells is unclear. These cells may be involved in the humoral response against tumor. I have found in preliminary studies that tumor-bearing animals generate a significant humoral response against multiple antigens including a 185 kDa protein that is most likely neu (data not shown). Alternatively, IL-4-secreting cells may represent immunosuppressive cells which could interfere with tumor eradication. I have began to explore immunosuppression as a mechanism by which tumors continue to grow despite infusion of tumor-specific T cells. I evaluated for the presence of CD4/CD25 regulatory T cells (Tregs) associated with tumors. To do this, I injected tumor into the peritoneal space and after 10 days harvested ascites fluid associated with the tumor (Fig. 5). The cells were examined by flow cytometry for dual expression of CD4 and CD25. Tregs were associated with both MMC and ANV tumors and constituted approximately 32% and 61% of the tumor-associated lymphocytes, respectively. These results suggest that one potential reason that T cells do not completely prevent tumors from growing is the presence of inhibitory Tregs. Since my interests are in the direct application of adoptive T cell therapy to human HER-2/neu-overexpressing breast cancer, I have begun to alter my model to more adequately reflect human breast cancer. Specifically, patients with advanced stage HER-2/neu-overexpressing breast cancer are being treated with trastuzumab, a HER-2/neu inhibitory monoclonal antibody. I have identified a similar monoclonal antibody active in the mouse 7.16.4. This antibody when infused into tumor-bearing animals is able to inhibit or stall progression of tumor similar to the effects of trastuzumab in humans (Fig. 6). Studies can be designed to use T cell therapy in tumor-bearing animals undergoing monoclonal antibody therapy. To study trafficking of T cells to tumor site, I have also developed a protocol for labeling that does not impact the function of the cells, as assessed by ELIspot analysis, for at least 48 hours after labeling (Fig. 7). T cell lines specific for neu helper peptide p781 were generated as described in Specific Aim 1 and labeled with varying concentrations of ^{111}Indium-oxine (0, 1, 3, and 6 μCi/million cells) for 45 minutes. The cells were then examined by ELIspot and it was found that antigen-specific T cell function was retained at all the doses of radiolabel used when compared to cells without label (i.e. zero concentration of indium on the graph) (Fig. 7A). I also examined whether dose of radiolabel affects uptake into the cells (Fig. 7B). It was observed that at a concentration of 1 μCi only about 30% of the label was taken up. The uptake increased as a function of dose and peaked at or below 3 μCi with a maximal uptake of the added dose.

3. To determine the feasibility of the expansion of HER2-specific CD4+ and CD8+ T cell lines from patients previously immunized with a HER2 peptide-based vaccine. The studies in this funding period have focused on the use of CD3/CD28 beads for rapid expansion following antigen-specific enrichment (Fig. 8A). This method successfully induced the rapid expansion of T cells as shown in Figure 8B. Using a method with whole CMV antigen and IL-2 during enrichment, it was observed that antigen specificity can be maintained at or above the levels measured in pre-expansion cultures (Fig. 9A). The resulting cells can be added back to autologous PBMC to improve antigen specificity (Fig 9B). If CMV was not present in the cultures during enrichment, CMV antigen-specificity drops by greater than 65% following expansion (data not shown). The inclusion of various cytokines during the enrichment phase is also being examined based on my previous

findings studying IL-12 and IL-15. I observed that IL-12, but not IL-15, was able to enhance the growth rate of cells during bead stimulation (Fig. 10A). However, the inclusion of IL-15, but not IL-12, significantly enriched for CD8 T cells specific for an MHC class I binding epitope derived from the flu matrix peptide (Fig. 10B). These results suggest that IL-15 may be a cytokine appropriate for expanding CD8 T cells. I am currently examining the effects of the inclusion of IL-12 or IL-15 on expansion of CD4 T helper cells using helper peptides as antigens. My preliminary findings suggest that IL-12 may be the cytokine of choice for CD4 T helper cells Based on my findings, our group have written a clinical trial for the infusion of T cells specific for HER2 which has been approved by our local IRB and the University of Washington Clinical Research Center. Batch records and standard operating procedures are currently being prepared for the T cell infusion trial.

Key Research Accomplishments:

- An neu antigen-negative variant tumor cell line was developed to enhance the breast cancer model of adoptive T cell therapy.
- T cell lines can be established using 3 different helper peptides derived from rat neu.
- Th1 T cell lines specific for neu oncoprotein are partially effective at inhibiting tumor growth in neu-transgenic mice.
- Th1 T cell lines derived from neu-tg mice are equally effective as lines derived from the parental FVB/N mice suggesting similar T cell avidity.
- Regulatory T cells associate with tumor which may reduce the efficacy of adoptive T cell therapy.
- Monoclonal antibody therapy was adapted to the model to reflect the current clinical setting in human HER-2/neu-overexpressing breast cancer.
- Antigen-specificity can be maintained in cells expanded non-specifically with anti-CD3/CD28 beads when an antigen-specific enrichment step is used in prior to expansion.
- Cytokines such as IL-15 can be used to augment low-level antigen-specific responses during *ex vivo* expansion.

Reportable outcomes:

Publications: (Published or submitted pertaining to the application during the funding period (April, 2002-April, 2003))

1. Knutson KL and Disis ML, 2002, Clonal diversity of the T cell repertoire responding to a dominant HLA-A2 epitope of HER-2/neu after active immunization in a patient with ovarian cancer. *Human Immunology* 63:547-57.
2. Knutson KL, Curiel TJ, Salazar L, and Disis ML, 2003, Immunologic Principles and Immunotherapeutic Approaches in Ovarian Cancer, Hematology/Oncology Clinics of North America. Editors: Holland and Disis, WB Saunders (in press).
3. Disis ML, Knutson KL, Salazar L, Schiffman K, 2003 Immunology and Immunotherapy, Breast Cancer Lippman (in press).
4. Knutson, K.L., Bishop, M.R., Schiffman, K., and Disis, M.L.: Immunotherapy of Breast Cancer. Cancer Chemotherapy and Biologic Response Modifiers- Annual 20. Editors: Giaccone, Schilsky and Sondel; Elsevier Science (20:351-69), 2001.
5. Knutson KL, Almond B, Mankoff DA, Schiffmann K, and Disis ML, 2002, Adoptive T-cell Therapy for the Treatment of Solid Tumors. Expert Opinion on Biological Therapy 2:55-66.
6. Bernhard H, Salazar L, Schiffman K, Smorlesi A, Schmidt B, Knutson KL, Disis ML, 2002, Active immunization against the HER-2/neu oncogenic protein. Endocrine-Related Cancer 9:33-44.
7. Knutson KL, Almand B, Ahlquist RM, and Disis ML, Neu-specific antibody therapy results the generation of antigen-loss variants in the neu-transgenic mouse (Submitted).
8. Knutson KL and Disis ML, IL-12 enhances the function and number of tumor antigen-specific Th1 lymphocytes during ex vivo expansion (Submitted).
9. Knutson, KL, Smorlesi, A, and Disis, ML, A Murine Neu-Transgenic (neu-tg) Breast Cancer Tumor Cell Line Established *In Vitro*, Expresses Class I and II MHC and Retains Expression *In Vivo*. (Submitted).
10. Almand, BA, Ahlquist, RM, Mankoff, DA, Disis, ML, and Knutson KL, 2002, Application of imaging approaches to the monitoring of targeted therapies for breast cancer. *Proc. AACR* 44:871 (Abstract #3807).
11. Knutson KL, Ahlquist RM, Almand, BA, and Disis, ML, 2002, Identification of helper peptides of rat neu that can mediate partial therapeutic anti-tumor responses in tumor-bearing neu-transgenic mice. *Proc. AACR* 44:873 (Abstract #3818).

12. Almand, BA, Ahlquist, RM, Disis, ML, and Knutson KL, 2002, Neu-specific antibody therapy results in tumor inhibition without receptor down-modulation in the neu-transgenic mouse. *Proc. AACR* 44:874 (Abstract #3819).
13. Disis ML, Faith M. Shiota, and Douglas G. McNeel, Knutson KL, 2003, Soluble Cytokines Can Act as Effective Adjuvants in Plasmid DNA Vaccines Targeting Self Tumor Antigens. *Immunobiology* 207:1-8.
14. Salazar LG, Fikes J, Southwood S, Ishioka G, Knutson KL, Gooley TA, Schiffman K, and Disis ML, Immunization of cancer patients with HER-2/neu-derived peptides demonstrating high affinity binding to multiple class II alleles (Submitted).

Research opportunities: As a result of the success of this research project, I have been promoted to Assistant Professor at the University of Washington. The position will be effective July, 2003.

Funding applied for: Data resulting from this award has been used to apply and obtain funding:

Title of project: *Ex Vivo Expansion of HER-2/neu specific T helper cells.*
PI: Keith Knutson, Ph.D.
Agency: NCI
Status: Pending (Priority Score 171)
Period: 2003-2008
Type: K01
Annual Direct Cost/% Effort: $121,350/80%
This grant outlines animal preclinical studies, human in vitro studies, for the development of T helper cell (Th1 and Th2) therapy for HER2 expressing advanced stage cancer.

Title of project: Multi-Antigen Vaccines for Breast Cancer
PI: Mary L. Disis, MD
Co-PI: Keith Knutson, PhD
Agency: NCI
Status: Active
Period: 2003-2007
Type: R01
Annual Direct Cost/% Effort: $225,000/20%
The overall goal of this proposal is to develop multi-antigen cancer vaccines targeting immunogenic overexpressed self-proteins that are biologically relevant to the growth of breast cancer and are involved in the malignant transformation. The utility of breast cancer vaccines will be in the immunization of breast cancer patients at high risk of relapse after optimal therapy or, eventually, immunization of individuals at high risk of developing breast cancer.

Title of project: The identification of tumor rejection antigens induced via epitope spreading in patients with breast and ovarian cancer after active immunization
PI: Keith L. Knutson, Ph.D.
Agency: NCI
Status: Pending
Period: 2003-2008
Type: R21
Annual Direct Cost/% Effort: $100,000/25%
The goal of this proposal is to identify tumor rejection antigens induced via epitope spreading in patients with breast and ovarian cancer after active immunization.

Title of Project: Vaccination Against Ovarian Carcinoma
PI: Karl-Erik and Ingegerd Hellstrom
Co-PI: Keith L. Knutson, Ph.D.
Agency: NCI
Status: Pending
Period: 2003-2008
Type: R01
Annual Direct Cost/% Effort: $75,000/20%

The goals of the proposal are to: (1) determine whether tumors transfected with murine CD137 scFv can prime tumor specific T cells in vitro using syngeneic tumors from neu transgenic mice, (2) determine whether tumors transfected with murine CD137 scFv can mediate an intraperitoneal antitumor response in vivo, and, (3) determine whether human ovarian tumors can be transfected with human CD137 scFv and can prime human tumor specific T cells in vitro.

Title of project: The identification of breast tumor antigens targeted by the immune response during tumor rejection
PI: Keith L. Knutson, Ph.D.
Agency: DOD
Status: Pending
Period: 2003-2004
Type: Concept
Annual Direct Cost/% Effort: $75,000/25%
The goal of this proposal is to identify tumor rejection antigens induced via epitope spreading in patients with breast and ovarian cancer after active immunization.

Project generated resources: I will submit the ANV and MMC cell lines to ATCC.

Conclusions:

In my previous funding period I were able to define strategies to generate Th1 neu-specific T cells that were able to mediate partial protection. During the current funding period, I have confirmed these findings and demonstrated that the T cell lines generated with the peptides were specific for neu-overexpressing tumors and could not lyse antigen-negative syngeneic tumors. I have began to identify methods of improving the outcome of adoptive T cell therapy. My efforts are focusing on the generation of a polyclonal response which has a more pronounced ability to eradicate tumor cells. I have also identified that some of the lymphocytes that ordinarily associate with tumor are regulatory T cells which can reduce the efficacy of adoptive T cell therapy. Understanding how to eliminate endogenous immunosuppression will likely improve my strategy. Recent studies by Drs. Dudley and Rosenberg at the NCI suggest the induction of lymphopenia prior to adoptive T cell may enhance the function of adoptively transferred T cells. While the mechanism of this effect is still unclear, a leading theory is that immunosuppressive regulatory T cells are reduced to levels that facilitate expansion and anti-tumor activity of transferred T cells. The widespread use of Herceptin will have a great impact on the translation of HER-2/neu targeted therapies to the clinic. I am beginning to adapt my adoptive T cell therapy to reflect changes in the treatment of patients with HER-2/neu-overexpressing cancers. I have improved our understanding of how to rapidly expand human antigen-specific T cells that maintain polyclonality with the use of IL-12, IL-15 and anti-CD3/CD28 beads. These findings are being applied to a clinical trial of adoptive T cell therapy of HER-2/neu-overexpressing tumors in advanced stage breast cancer patients.

Figures:

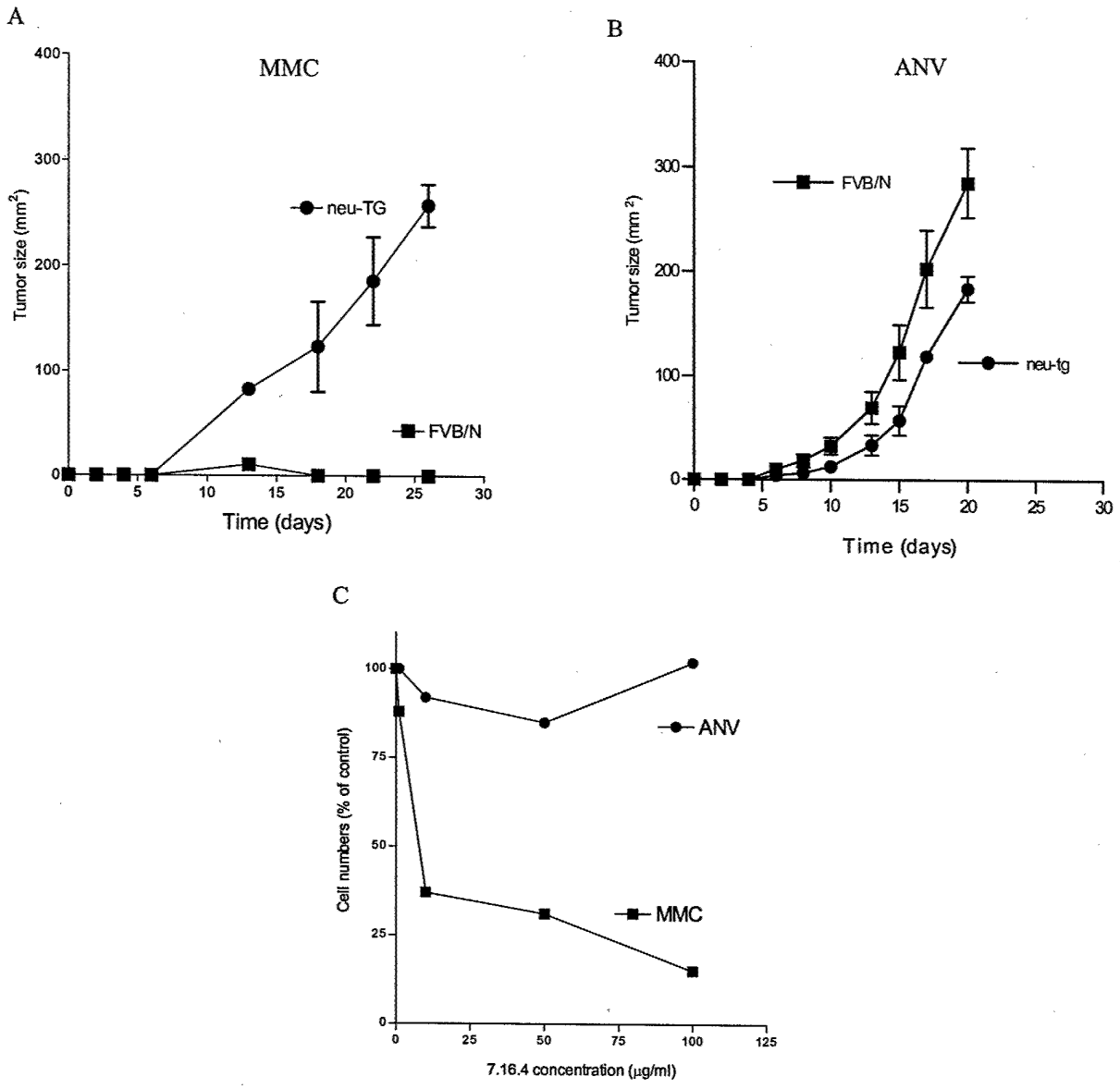


Figure 1. A neu-negative mammary tumor cell line, ANV, was established *in vitro* from an antigen-escape variant. A. Shown is the growth of neu-expressing MMC in either neu-Tg (circles) or parental FVB/N (squares). B. Shown is the growth of neu-negative ANV in either neu-Tg (circles) or parental FVB/N (squares). Each data point shows the mean and SEM of 3 mice. C. Shown are the effects of a neu-specific inhibitory monoclonal antibody on the growth of neu-expressing MMC and neu-negative tumor cells. Each data point represents the mean (±SEM) of triplicate determinations.

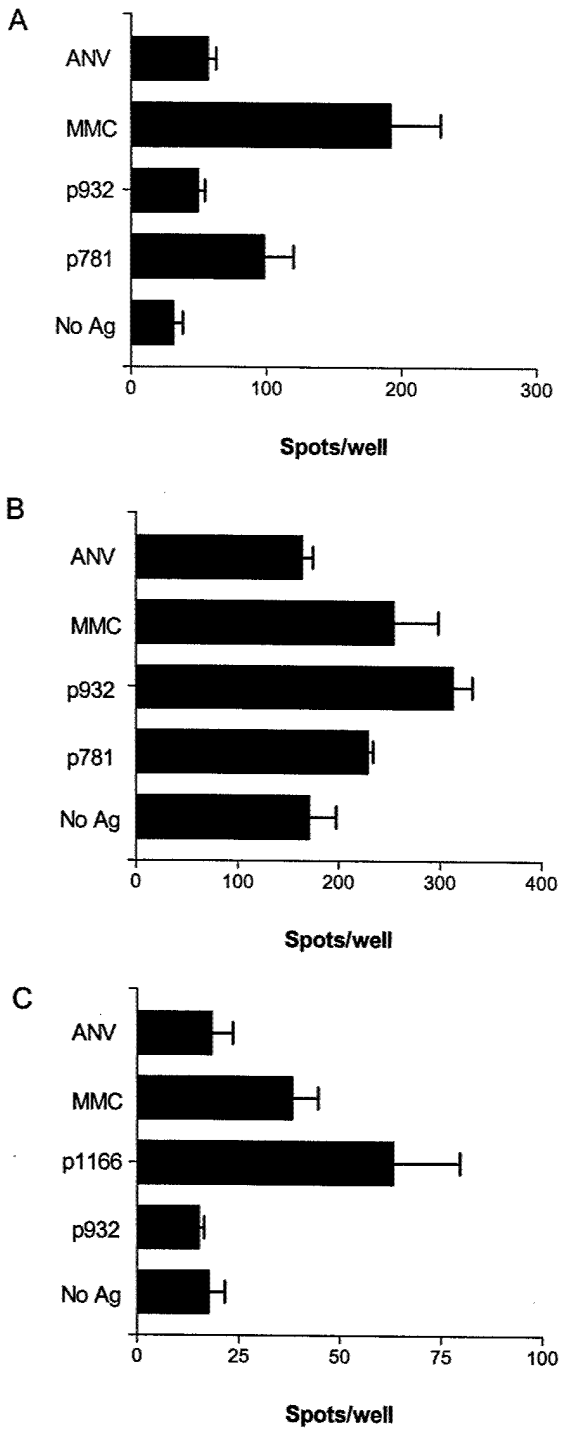


Figure 2. Tumor antigen specific T cell lines generated *in vitro* are specific neu-overexpressing tumors. Shown are the IFN-γ ELIspot responses of a representative p781- (Panel A), p932- (Panel B), and p1166-specific (Panel C) T cell lines to peptide antigens and murine breast tumor cell lines. The data are presented as the number of spots observed per well. Each data point is the mean (±SEM) of 6 replicates. MMC are neu-overexpressing tumor cells and ANV are neu-negative tumor cells. Both tumor cell lines are derived from mammary tumors in neu-tg mice.

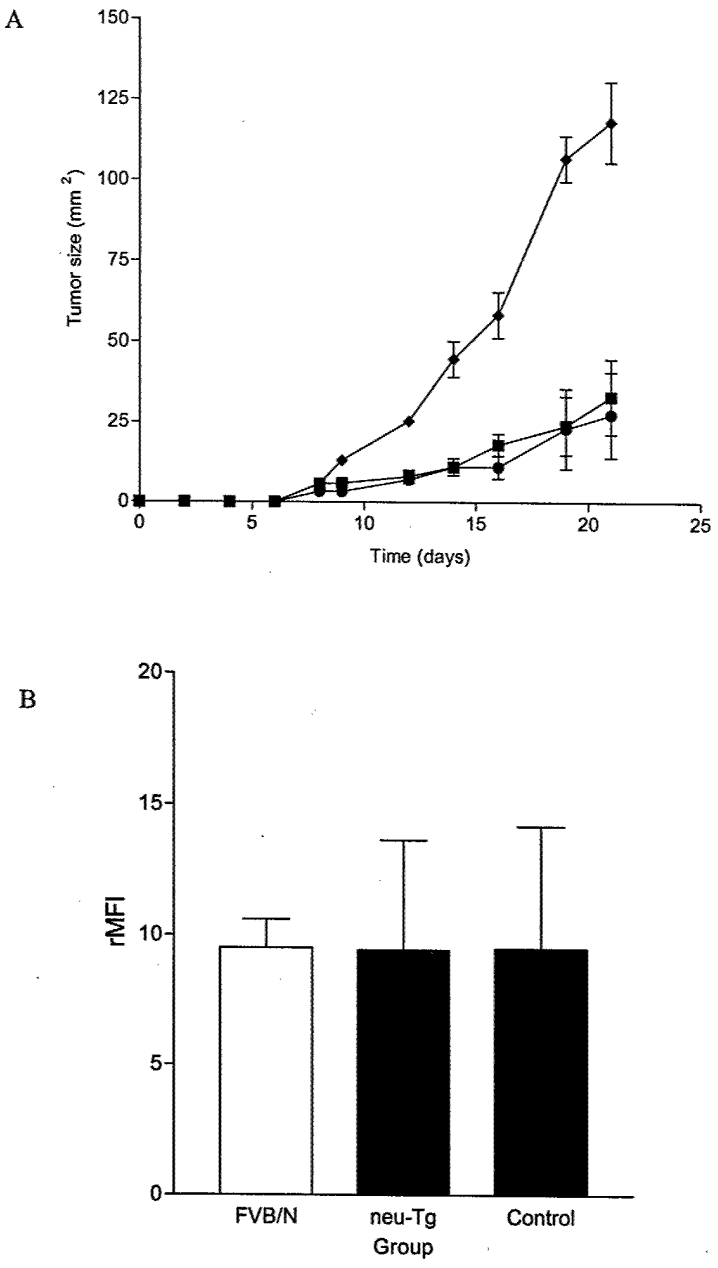


Figure 3. T cells specific for rat neu peptide p1166, expanded *ex vivo*, from either neu-tg or parental FVB/N can be partially therapeutic *in vitro*. (A) 6 x 10^6 neu-expressing MMC tumor cells were injected on day 0. One day later, 6 x 10^6 p1166-specific T cells (circles or squares) or normal neu-tg splenocytes (control, diamonds) were infused and tumor growth was monitored. The p1166-specific T cells were derived from either neu-tg or parental FVB/N mice. Each data point represents the mean (±SEM) of measurements from 3 mice. (B) Tumors derived from the mice in panel A were stained with anti-neu antibodies. The average (±SEM) mean relative staining intensity (rMFI) is shown for each group.

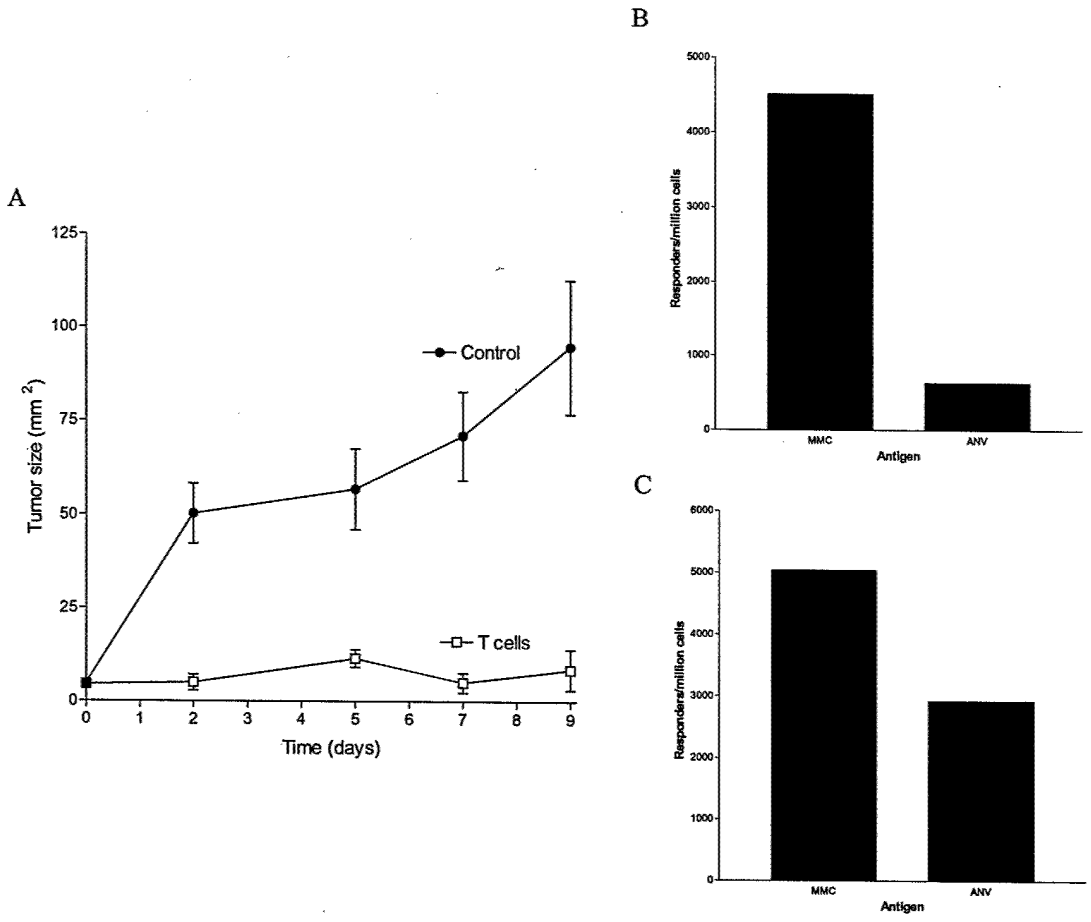


Figure 4. Polyclonal T cell lines completely inhibit tumor growth. A. 6 x 10^6 neu-expressing MMC tumor cells were injected on day 0. One day later, 6 x 10^6 T cells or normal neu-tg splenocytes (control, diamonds) were infused and tumor growth was monitored. The T cells were derived from parental FVB/N mice. Each data point represents the mean (±SEM) of measurements from 5 mice. Shown are the IFN-γ (panel B) or IL-4 (panel C) ELIspot responses of tumor-specific T cell lines in response to neu-expressing MMC or neu-negative ANV. The data are presented as the mean number of spots calculated from 6 replicates.

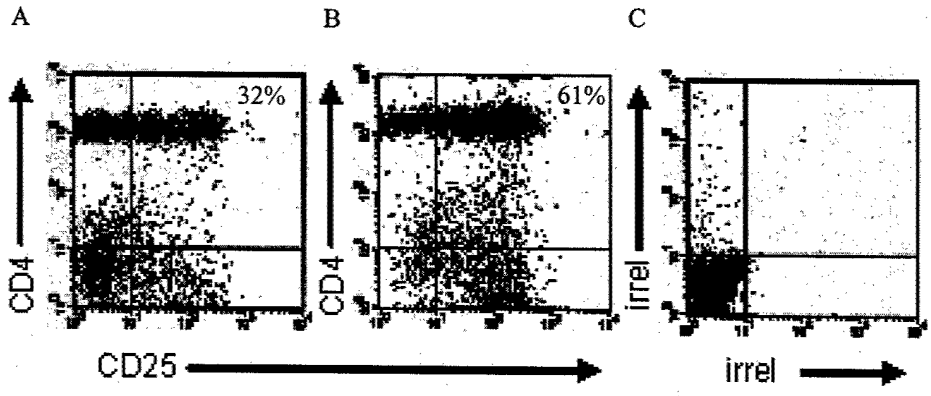


Figure 5. CD4/CD25+ T regulatory cells associate with both MMC and ANV mammary tumor cells. T cells derived from ascites fluid of mice bearing peritoneal tumors were evaluated by flow cytometry. T cells were dual-stained with anti-CD4 and anti-CD25 antibodies. CD25 intensity is shown on the X-axis for panels A and B. CD4 intensity is shown on the Y-axis for panels A and B. Irrelevant fluorescence is shown in panel C. Panels A and B depict lymphocytes derived from MMC and ANV tumor-bearing animals respectively. The value in the upper right quadrant represents the percentage of the total cells that the cells in this quadrant constitute.

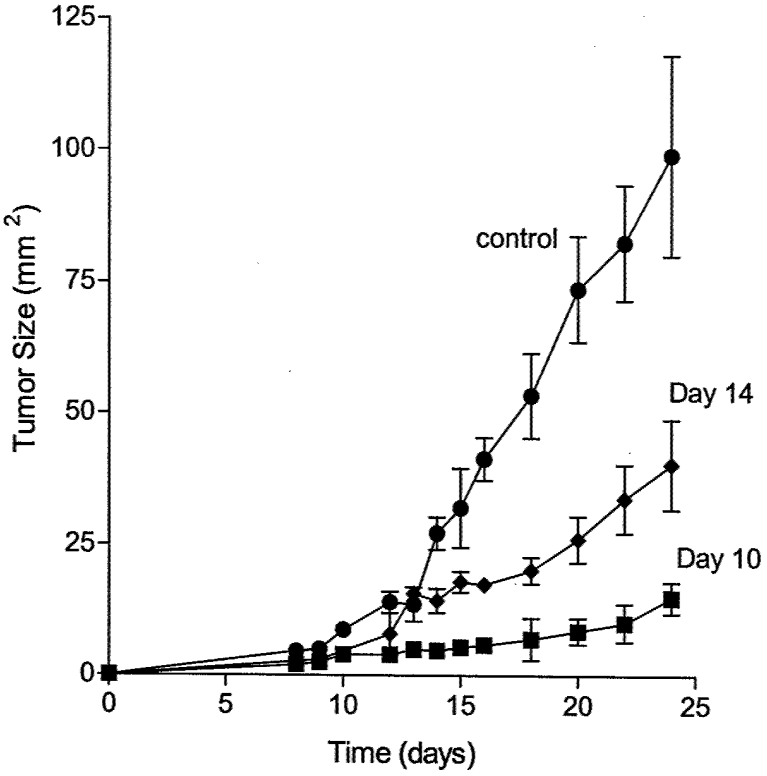


Figure 6. Neu-specific monoclonal antibody inhibits neu-mediated tumor growth *in vivo* in an established tumor setting. Shown are tumor measurements from tumor-bearing control mice (PBS, circles) and tumor-bearing mice treated with 7.16.4 antibody starting on either day 10 (squares) or day 14 (diamonds) following tumor cell injection. Each data point is the mean tumor measurements (±SEM) from 15-16 mice.

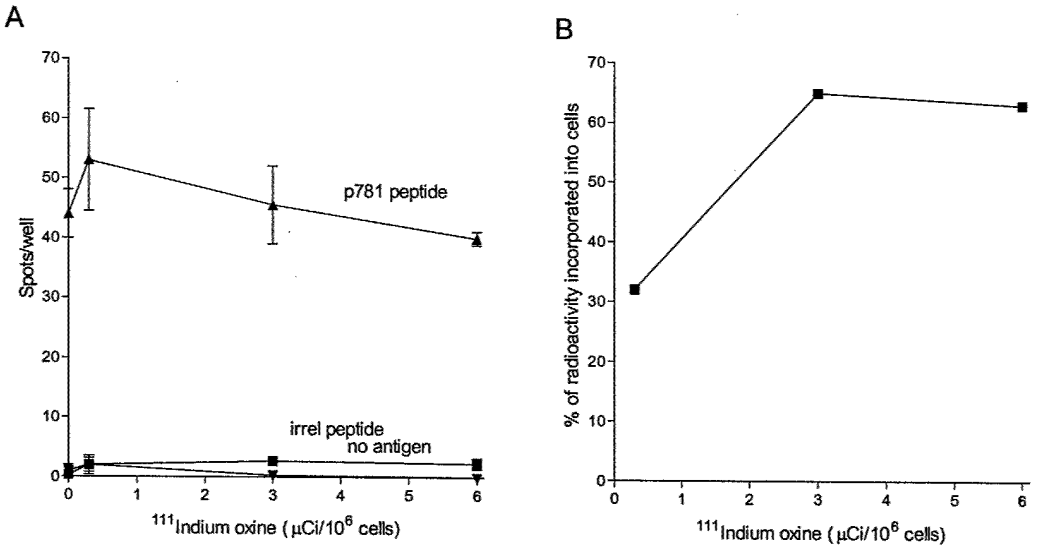
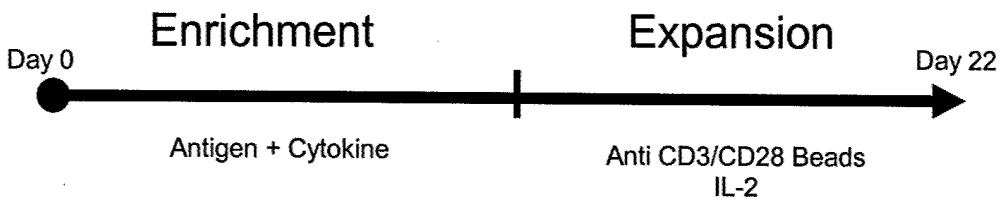


Figure 7. Neu-specific T cells can be radiolabeled without loss of function. Panel A shows the percent of label associating with the cells following 45 minutes of labeling a neu-specific T cell line with varying (0, 30, 300, 600 $\mu Ci/10^8$ cells) concentrations of ^{111}Indium-oxine. Panel B shows IFN-γ secretion results using ELIspot assay following labeling of a T cell line specific for the p781 helper peptide of neu. The T cell line was labeled with varying concentrations of ^{111}Indium-oxine and stimulated *in vitro* with p781 peptide (circles), an irrelevant neu helper peptide (irrel, triangles) or no antigen (squares). ELIspot analysis was performed. Each symbol is the mean (±SEM) of 3 replicates.

A



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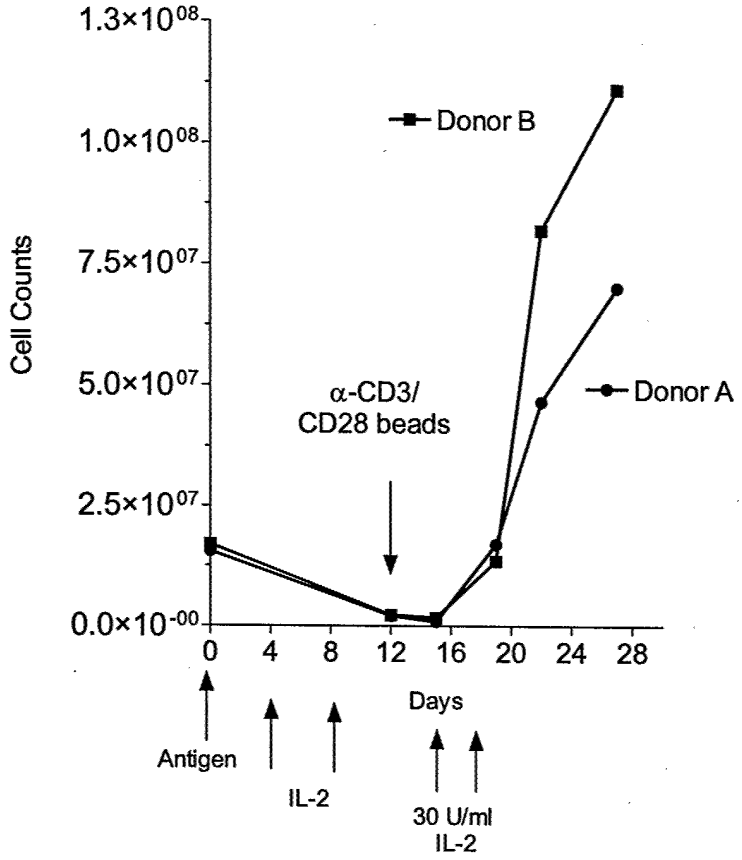

Figure 8. T cells can be expanded with anti CD3/CD28 beads. An expansion scheme was established that has two phases (Panel A). The first phase is an antigen enrichment period in which antigen-specific T cells are selected. The second phase is a rapid expansion phase in CD3/CD28 beads. In the lower graph (Panel B)is the growth results of T cells from 2 donors taken thru the expansion scheme.

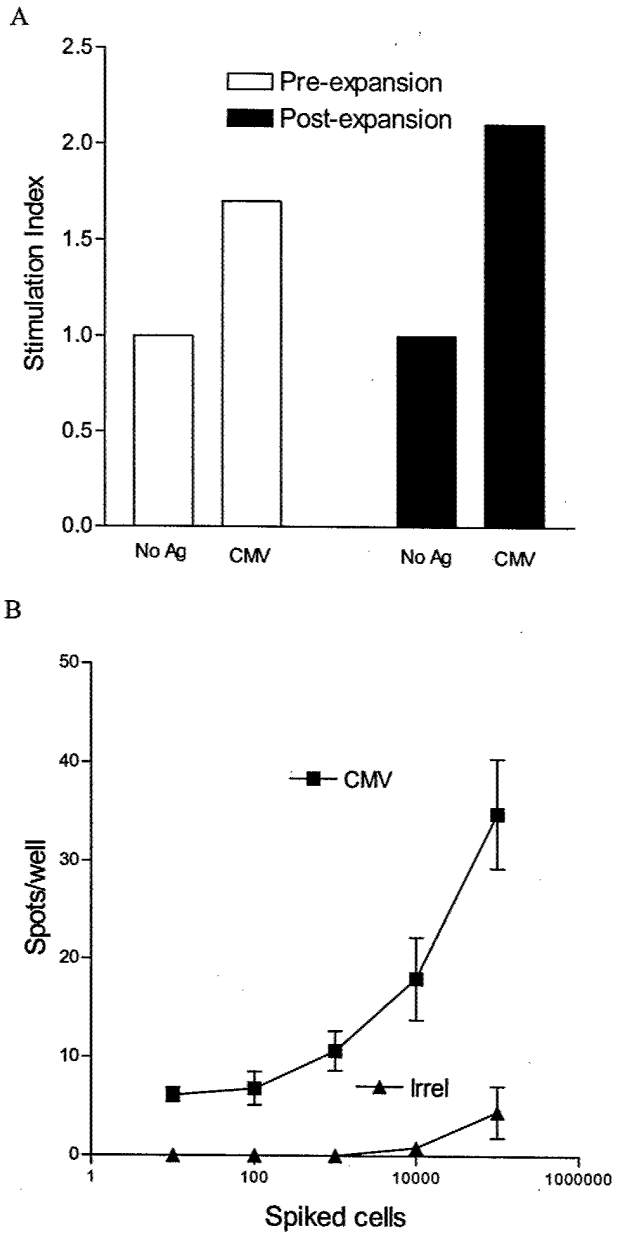

Figure 9. Antigen-specificity is preserved following *ex vivo* expansion. Cells were taken through the *ex vivo* expansion described in Figure 8. The antigen used was CMV lysate. Following enrichment and bead expansion, the cells were restimulated with or without CMV and proliferation was measured with thymidine incorporation assays (Panel A). Data is shown as the mean of 6 replicates and compared with the response observed in pre-expansion samples. Panel B shows that expanded CMV specific T cells can reconstitute CMV activity in normal PBMC. CMV-specific T cells lines were added into normal viable PBMC at different doses and the lines were tested for reactivity against CMV using ELIspot. Results shown are the mean (±SEM) of triplicate determinations.

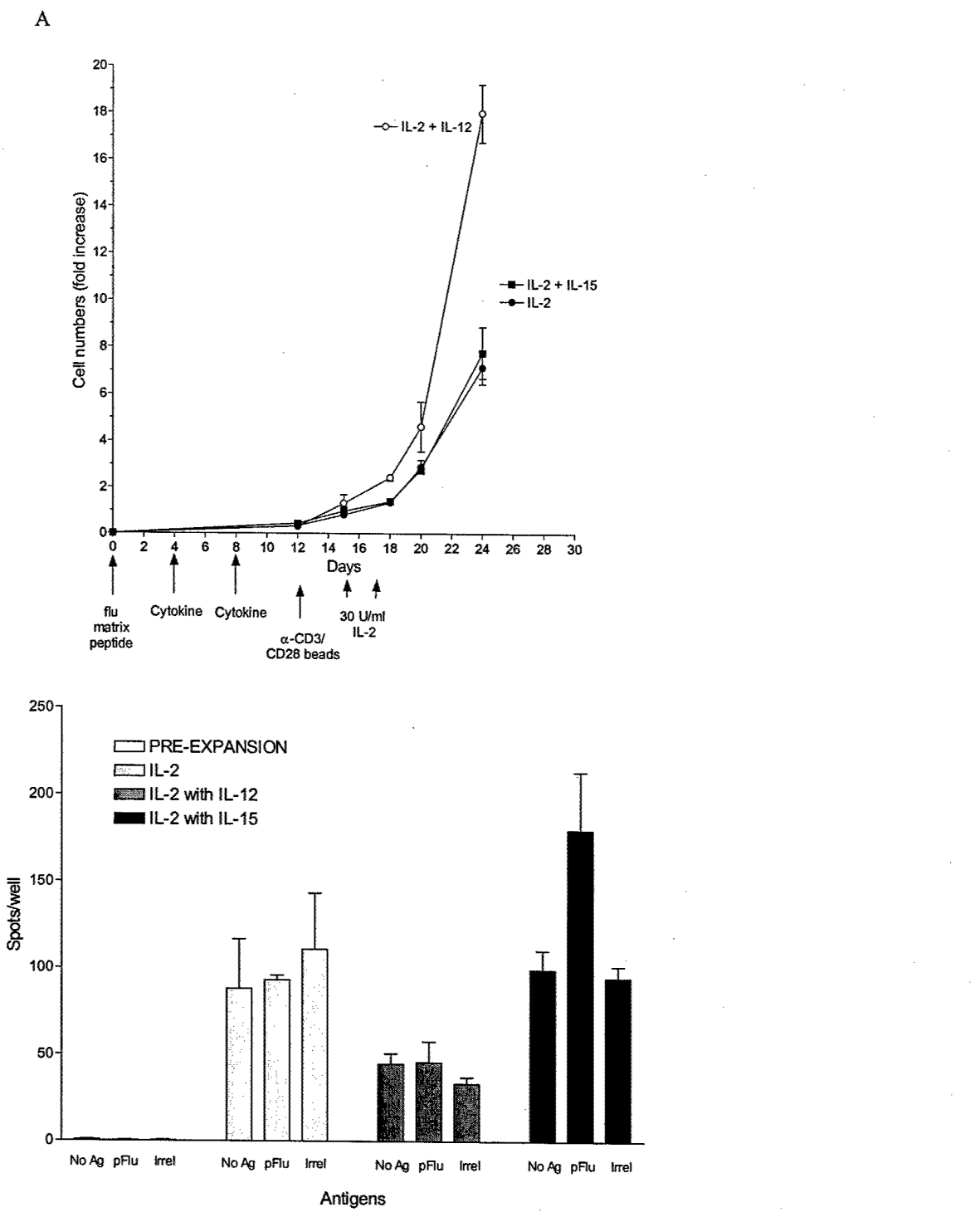

Figure 10. Inclusion of cytokines during enrichment phase can enhance low-level CD8 peptide-specific T cells. Cells were taken through the *ex vivo* expansion described in Figure 8. The antigen used was an HLA-A2 flu matrix peptide. During the enrichment phase cells received either IL-2 alone or IL-2 in combination with IL-12 or IL-15. Panel A shows the effects of cytokines on the growth of T cells during *ex vivo* expansion. Panel B shows the specificity for flu matrix peptide as assessed by ELIspot. Each T cell culture was restimulated at the end of the culture period with flu matrix antigen, an irrelevant HLA-A2 antigen, or no antigen. The results shown are the mean (±SEM) of 6 replicates. Only IL-15 was able to enhance the precursor frequency of flu-matrix peptide specific T cells.

ELSEVIER

Clonal Diversity of the T-Cell Population Responding to a Dominant HLA-A2 Epitope of HER-2/neu After Active Immunization in an Ovarian Cancer Patient

Keith L. Knutson, and Mary L. Disis

ABSTRACT: Natural antigen processing and presentation of antigen is thought to be important for the generation of a broad functional repertoire of antigen-specific T cells. In this study, the T-cell repertoire to an immunodominant human leukocyte antigen A2 (HLA-A2) binding peptide epitope of HER-2/neu, p369-377, was examined in a patient following immunization with a peptide-based vaccine consisting of helper peptides encompassing HLA-A2 peptide epitopes. The responding T-cell repertoire generated was both phenotypically and functionally diverse. A total of 21 p369-377 clones were generated from this patient. With the exception of two clones, all clones were $CD3^+$. Sixteen of the clones were $CD8^+/CD4^-$. Five of the clones were $CD4^+/CD8^-$, despite being generated with an HLA-A2 binding peptide. Nineteen of 21 of clones expressed the αβ-T-cell receptor (TCR). The remaining two clones expressed the γδ T-cell response (TCR). Selected αβ-TCR clones, both $CD8^+$ and $CD4^+$, could lyse HLA-A2 transfected HER2 over-expressing tumor cells and p369-377-loaded B-lymphoblastic cell line. In addition to their lytic capabilities these clones could be induced to produce interferon-γ (IFN-γ) specifically in response to p369-377 peptide stimulation. The 2 γδ-TCR clones expressed CD8 and lysed HLA-$A2^+$ HER-2/neu^+ tumor cells, but not HLA-$A2^-$ HER-2/neu^+ tumor cells. One of γδ-TCR clones also released IFN-γ directly in response to p369-377 stimulation. These results suggest that a tumor antigen TCR, directed against a specific epitope, can be markedly polyclonal at multiple levels including CD4/CD8 and TCR. *Human Immunology 63, 547–557 (2002).* © American Society for Histocompatibility and Immunogenetics, 2002. Published by Elsevier Science Inc.

KEYWORDS: vaccination; T lymphocytes; CTL; tumor immunity; γδ T cells

ABBREVIATIONS

BLCL B-lymphoblastic cell line
TCR T-cell receptor
CTL cytolytic T lymphocyte

INTRODUCTION

One of the most common vaccination strategies used to generate T cells specific for tumor antigen is to use peptides formulated to bind directly to human leukocyte antigen (HLA) molecules eliminating the need for intracellular processing and presentation of antigen. Vaccination with peptides is advantageous compared with other methods, such as using DNA or proteins, for several reasons including the fact that peptides are easily constructed and can be chosen to target specific T-cell subsets, such as cytolytic T cells (CTL) [1–3]. Vaccination using HLA class I binding peptides have been tested most often because of the availability of HLA class I sequence information and the observations that CTL are critical for antitumor immunity.

Some clinical studies using vaccination with HLA class I binding peptides have reported that peptide-specific precursor frequencies can become elevated, but that these peptide-specific T cells may not recognize naturally processed antigen [4]. The lack of recognition of antigen presented in the major histocompatibility complex (MHC) may be due to the fact that the peptides, usually constructed from motif-based algorithms, were not naturally processed. Alternatively, the high pharmacologic levels of peptides usually administered in a vac-

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cine could have elicited a T-cell repertoire that cannot respond to the low levels of peptides presented naturally. Thus, it is becoming apparent that natural levels of presentation of peptide antigen, delivered through antigen processing pathways, may be better suited to generate an immune response when using peptide-based vaccines. The levels of endogenously processed antigen presented by antigen presenting cells is a function of many factors including the antigen load as well as the types and amounts of proteasomes present [5]. Several strategies have been developed to deliver peptides to the inside of the cell where natural presentation can be achieved, including mini-gene epitope delivery [6] or fusing the epitopes with molecules designed to deliver the peptides to the endosomal pathway, such as lysosomal membrane associated protein [7]. Another strategy is to deliver the HLA class I peptides that are fully contained within longer helper epitopes, which can result in natural presentation of the encompassed class I peptides [8].

In this study, we examined the phenotypic and functional diversity of the T-cell repertoire to HER-2/neu peptide p369-377 in a HLA-A2 patient with a HER-2/neu-overexpressing ovarian cancer. The patient had been previously immunized with a HER-2/neu helper peptide vaccine, which contained the helper peptide p369-386 [8]. This helper peptide encompassed the HLA-A2 peptide, p369-377. Therefore, the resulting p369-377-specific T-cell repertoire produced *in vivo* was a function of natural processing of antigen.

MATERIALS AND METHODS

Patient

The patient was enrolled in a Phase I HER-2/neu peptide-based vaccine trial approved by the University of Washington's Human Subjects Division and the United States Food and Drug Administration, and had received definitive conventional therapy for her disease [8]. This Phase I clinical trial was designed to evaluate safety and immunologic responses to the vaccine. Furthermore, all of the patients had either no evidence of disease or minimal residual stable disease. Clinical responses to vaccine were not possible unless patients were followed for an extended time to relapse. The patient signed a protocol-specific consent and received monthly vaccinations with three 15 amino acid (15-aa) HER-2/neu-derived peptides (p369-386, p688-703 and p971-984) containing within each the putative HLA-A2 binding motifs p369-377 [9], p689-697 [10], and p971-979 [11]. The vaccine preparation was prepared and delivered as previously described [8]. The patient underwent peripheral blood draws prior to and 30 days following each vaccination for immunologic monitoring. Leukapheresis was obtained 30 days following the final vaccination for the generation of T-cell clones.

Materials

The following peptides used in this study, either for immunization or *in vitro* use, were as follows: HER-2/neu peptides, p369-386, KIFGSLAFLPESFDGDPA [12], p688-703, RRLLQETELVEPLTPS [12], p971-984, ELVSEFSRMARDPQ [12], p369-377, KIFGSLAFL, p1066-1074, SEEEAPRSP. All peptides used for *in vitro* immunologic assays were manufactured either by United Biochemical Inc. (Seattle, WA, USA) or Multiple Peptide Systems (San Diego, CA, USA) and all were > 95% pure as assessed by HPLC and mass-spectrometric analysis. Ficoll/Hypaque was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). RPMI-1640, HBSS, and PBS were purchased from Life Technologies (Rockville, MD, USA), and EHAA-120 was purchased from Biofluids (Rockville, MD, USA). The [^{3}H] thymidine and [^{51}Cr] sodium chromate were purchased from NEN Life Science Products (Boston, MA, USA), human AB+ serum from Valley Biomedical, Inc. (Winchester, VA, USA), sterile nitrocellulose-backed microfiltration 96-well plates from Millipore Corp (Bedford, MA, USA), and streptavidin-alkaline phosphatase and AP-colorimetric reagents were from BioRad (Hercules, CA, USA). Purified anti-IFN-γ (clone # 1-D1K) and biotin-conjugated anti-IFN-γ (clone # 7-B6-1) were purchased from Mabtech AB (Nacka, Sweden). Anti-CD8-FITC, anti-CD4-PE, anti-γδ-TCR, and anti-αβ-TCR antibodies were purchased from Pharmingen (San Diego, CA, USA). HLA testing was performed by the Puget Sound Blood Bank (Seattle, WA, USA).

Cell Lines

Epstein-Barr virus (EBV) transformed B-lymphoblastoid cell lines (BLCL) were produced from peripheral blood mononuclear cells (PBMC) using culture supernatant from the EBV-producing B95-8 cell line (American Type Culture Collection, Manassas, VA, USA). The HER-2/neu-overexpressing cell lines SKOV3 and SKOV3-A2, and BLCLs were maintained in RPMI-1640 with L-glutamine, penicillin, streptomycin, 2-mercaptoethanol, and 10% fetal calf serum. The SKOV3-A2 tumor cells are the same as SKOV3 tumor cells, except that they are stably transfected with a vector encoding HLA-A2 [13].

Preparation of PBMC and Cloning of Peptide-Specific T Cells

PBMC were isolated by density gradient centrifugation as previously described [12]. Cells were analyzed immediately or aliquoted and cryopreserved in liquid nitrogen in freezing media (90% fetal bovine serum and 10%

dimethylsulfoxide) at a cell density of 25 to 50×10^6 cells/ml until use. Antigen-specific T-cell clones were generated by culturing 25×10^6 PBMC in T25 tissue culture flasks in 20 ml of T-cell medium. The HLA-A2 peptide, p369-377, was added to the flasks to 1 μM. The flasks were incubated at 37 °C and 5% CO_2. On day 3 and every other subsequent day, IL-2 was added to 5 U/ml. On day 10, *in vitro* stimulation was performed with peptide-pulsed, irradiated autologous PBMC. The cultures were further incubated for an additional 10 days with periodic IL-2 administration. T-cell clones were derived from the T-cell lines after two *in vitro* stimulations. For cloning, bulk cultures were diluted to achieve approximately 0.3 viable cells/200 μl and plated onto four flat-bottom 96-well plates in complete medium. Peptide-pulsed, irradiated autologous PBMC (2.0×10^5) were added to each well in the presence of 50 U/ml IL-2. The wells were then tested for lytic activity in a ^{51}Cr release assay using 50 μl of cells from each well after 14 days. Positive wells were identified as those having specific activity of 5% or greater. The positive wells were transferred to new 96-well plates and subsequently restimulated with peptide-pulsed, irradiated autologous BLCL.

The cultures were eventually expanded and carried using IL-2 and peptide-pulsed, irradiated autologous BLCL.

T-Cell Proliferation Assays

HER-2/neu-specific T-cell proliferative responses were measured at baseline and at the end of the study. T-cell proliferation was assessed using a modified limiting dilution assay designed for detecting low frequency lymphocyte precursors based on Poisson distribution as previously described [12, 14]. HER-2/neu peptide-specific T-cell responses were measured at baseline and at the time of the final vaccination using freshly prepared PBMC. PBMC were prepared by Ficoll-Paque (Pharmacia AB, Uppsala, Sweden) centrifugation and resuspended in media consisting of equal parts of EHAA 120 (Biofluids, Inc.) and RPMI 1640 (Gibco, Grand Island, NY, USA) with L-glutamine, penicillin/streptomycin, β-2 ME, and 10% AB serum (ICN Flow, Costa Mesa, CA). PBMC were cocultured with 50 μg/ml of the various individual HER-2/neu peptides. Specifically, 2×10^5 PBMC/well were plated into 96-well round bottom microtiter plates (Costar, Cambridge, MA, USA) with antigen at 37 °C in an atmosphere of 5% CO_2 for 5 days. All antigens were tested in 24-well replicates. Eight hours before termination of culture, each well was pulsed with 1 μCi ^3H-thymidine (New England Nuclear, Wilmington, DE). The cultures were then harvested onto glass fiber filters and the incorporated radioactivity was measured with a Microbeta 1450 scintillation counter (Wallac). Peripheral blood T-cell response data presented here is expressed as a standard stimulation index (SI), which is defined as the mean of all 24 experimental wells divided by the mean of the 24 control wells (no antigen). An age-matched control population of 30 volunteer blood donors was analyzed similarly (data not shown). No volunteer donor had a response to HER-2/neu peptides. The mean and 3 standard deviations of the volunteer donor responses to all antigens (SI of 1.98) established a baseline, therefore an SI of > 2 was considered consistent with an immunized response.

Enzyme-Linked Immunosorbant Spot

An enzyme-linked immunosorbent spot (ELIspot) assay was used to determine precursor frequencies of peptide-specific T lymphocytes as previously described with some minor modifications [8]. On day 1, 2.5×10^4 viable cells, as determined by trypan blue staining were plated, in quadruplicate, into 96-well, anti-IFN-γ-coated nitrocellulose plates in 100 μl media. Because at 10 days following restimulation some irradiated PBMC and BLCL can still exclude trypan blue, the viable cell counts do not reflect the true numbers of each antigen-specific clonal population. Thus, our ELIspot analysis is only able to measure the numbers of cells responding to antigen and not the proportion of the antigen-specific T-cell clones that are responding with IFN-γ release. The cells were stimulated with 100 μl of media containing 2.0×10^5 autologous, irradiated (3000 rads) BLCLs prepulsed (1 h, RT) with or without antigen (10 μg/ml). The cells were further incubated for 20 h at 37 °C and detection of bound IFN-γ was performed as previously described [8].

[^{51}Cr]-Release Assays

Cytolytic activity was measured using standard 4-h[^{51}Cr]-release assays as previously described [8]. The percent specific activity was calculated using the following equation: % specific lysis = (sample well release − basal release)/(detergent release − basal release).

Flow Cytometry

Clones were harvested and washed in FACS staining buffer (PBS containing 20-mM glucose and 0.5% BSA). Cells were stained in 50-μl FACS staining buffer containing control antibody, anti-CD4, anti-CD8, anti-αβ-TCR, anti-γδ-TCR, or anti-CD3, for 1–2 h at 4 °C. Following two washes the cells were fixed in PBS containing 1% paraformaldehyde and analyzed by flow cytometry. Data presentation was completed using CellQuest flow cytometry software (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA).

RESULTS

Immunity to HER-2/neu peptide p369-386 is generated as a result of vaccination with helper peptides in an HLA-A2$^+$ ovarian cancer patient. T-cell proliferative responses were measured against 15-aa HER-2/neu peptides prior to, during, and following the vaccination series. As illustrated in Figure 1A, prior to immunization proliferative responses were not detected to any of the HER-2/neu peptides contained within the vaccine formulation. The stimulation index to p369-386 was 0.9, to p688-p703 was 0.9, and to p971-984 was 0.8. The patient demonstrated a proliferative response to tetanus toxoid (SI = 7.5). Following vaccination, 6 months after the initial immunization, a T-cell proliferative response was measured against 15-aa HER-2/neu peptide, p369-386 (Figure 1B). The stimulation index to p369-386 was 4.2, to p688-p703 was 1.4, and to p971-984 was 1.6. The tetanus toxoid response remained stable during the course of immunization and after the last vaccine was 4.9. The peptide, p369-386, encompassed within its sequence a defined HLA-A2 binding epitope, p369-377 [8, 15]. Twenty-one T-cell clones were isolated using this HLA-A2 peptide, representing a cloning efficiency of approximately 5%.

A polyclonal T-cell response could be elicited to p369-377, an HLA-A2 HER-2/neu epitope encompassed with the sequence of the helper epitope, after active immunization. The majority (19/21) of the clones were > 90% CD3$^+$. Nineteen of 21 clones expressed the αβ-TCR, and two clones expressed γδ-TCR (clones IDI and 3F7). Representative histograms of TCR staining for 2 αβ-TCR (example clones 2F10 and 2G2) and the 2 γδ-TCR T-cell clones are depicted in Figure 2. Although the majority of the monoclonal populations expressed CD8 (16/21), some clones expressed CD4 (5/21) despite being cloned using the HLA-A2 peptide, p369-377. Representative flow cytometry dot plots from 4 αβ-TCR clones, 2F10 (CD8$^+$), 2G2 (CD8$^+$), 2A2 (CD4$^+$), and 3G9 (CD4$^+$) stained with anti-CD8 and anti-CD4, are illustrated in Figure 3. Representative clones from each phenotypic group both αβ or γδ were chosen for evaluation in a more detailed fashion.

HER-2/neu peptide-specific αβ-TCR clones secrete IFN-γ in response to antigen. The αβ-TCR clones were examined for peptide-specific release of IFN-γ in an ELIspot assay (Figure 4). Representative αβ-TCR, CD8+ T cells were tested against p369-377, an irrelevant HER-2/neu 9 amino acid peptide, or no antigen. The number of spots detected in wells containing p369-377 was 137 ± 17 (mean ± standard error of mean [SEM]), 177 ± 22, and 102 ± 17, for the clones, 3H8 (CD8), 2G2 (CD8), and 2F6 (CD8) respectively. These means were significantly greater than the mean number

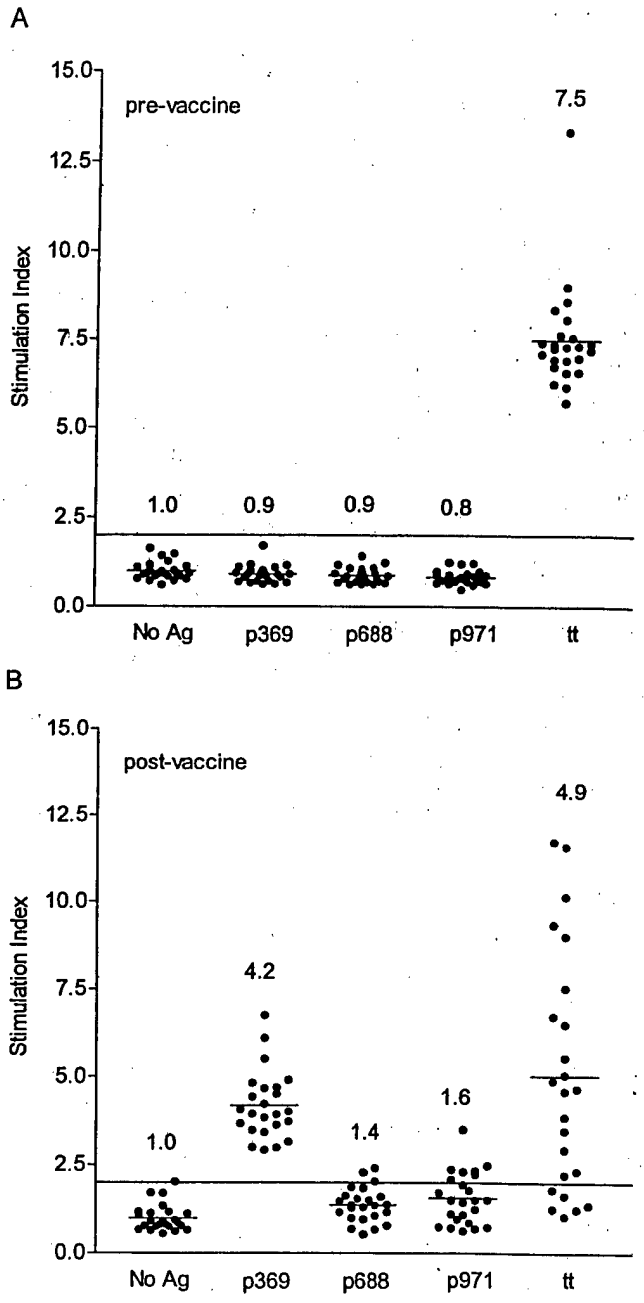

FIGURE 1 Immunity to HER-2/neu peptide, p369-386 is generated as a result of vaccination with helper peptides in a human leukocyte antigen A2 (HLA-A2$^+$) ovarian cancer patient. Depicted are the proliferation responses to media alone, the vaccine peptides, p369-386, p689-703, and p971-984. Tetanus toxoid (tt) was added as a positive control. Data from peripheral blood mononuclear cells drawn at the time of the first visit (Panel A) and the last immunization (Panel B) are illustrated. The long horizontal line spanning the width of the graph depicts average CPM of the no antigen wells + 3 standard deviation. The short horizontal lines within the data symbols represent the mean of the 24 replicates for each condition. The values above each data set are the calculated stimulation index.

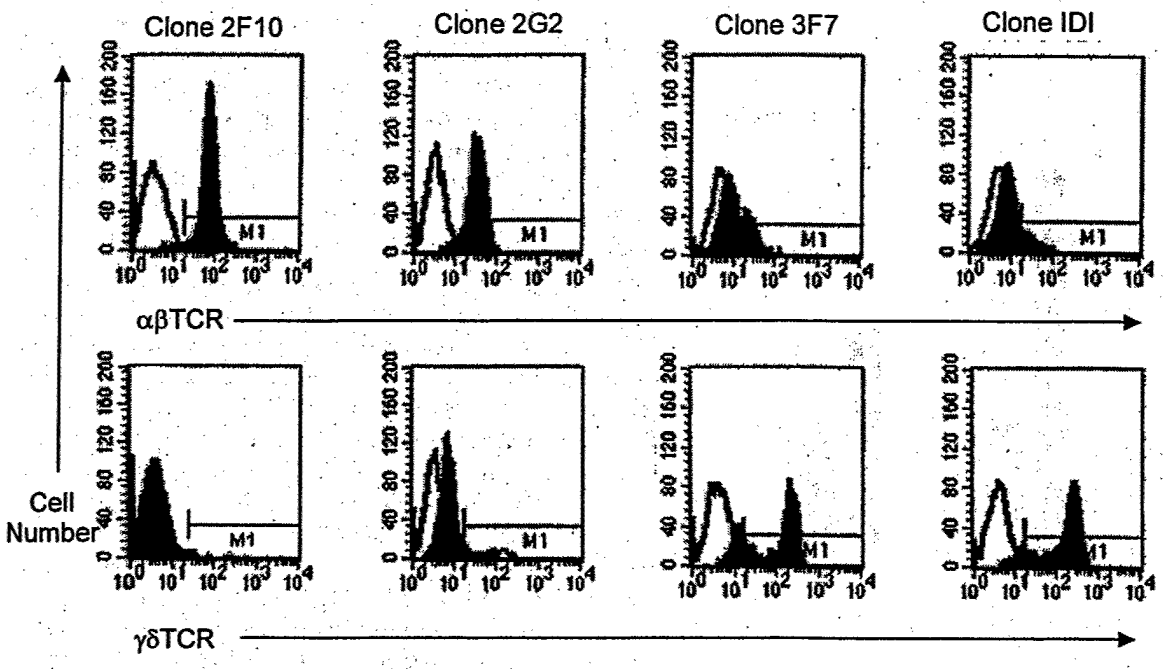

FIGURE 2 Both αβ T-cell reponse (αβ-TCR) and γδ-TCR clones were isolated following HER-2/neu peptide vaccination. Illustration is a representative flow cytometry analysis, staining for either αβ-TCR (top row) or γδ-TCR (bottom row) of select T-cell clones. The results are representative of two independent experiments yielding similar results. The fluorescence intensity is plotted on the x axis and the cell counts are plotted on the y axis.

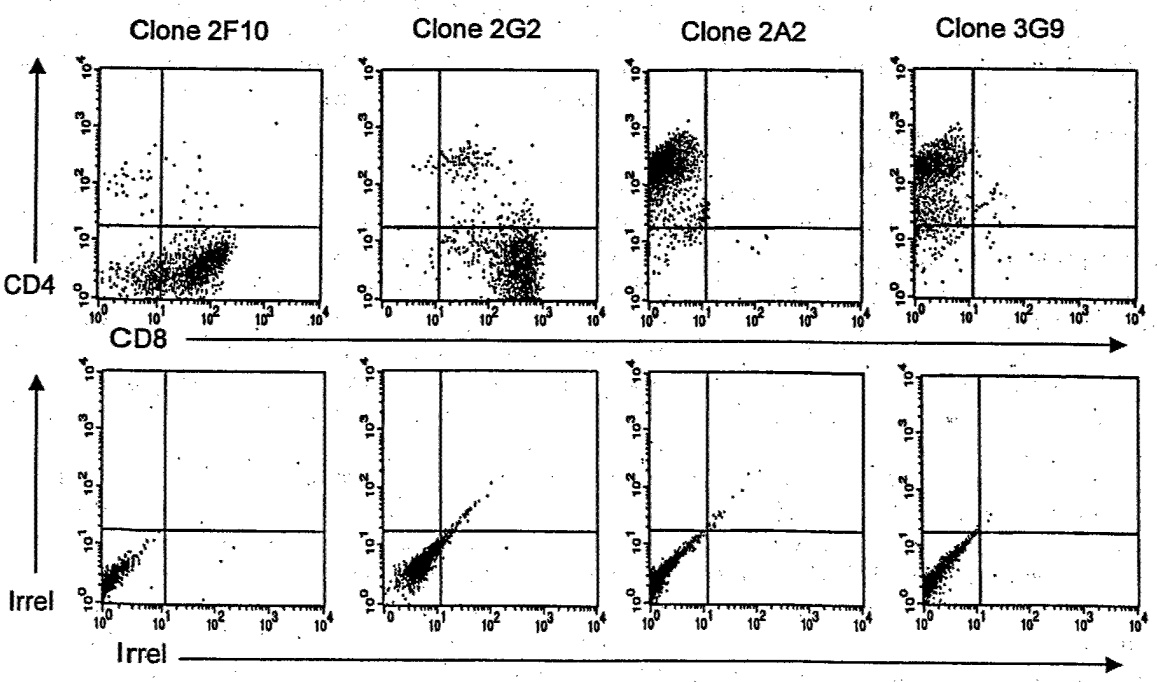

FIGURE 3 Both $CD4^+$ and $CD8^+$ clones were isolated following HER-2/neu peptide vaccination. Illustration is the representative flow cytometry data of p369-377-specific αβ T-cell reponse (αβ-TCR) T-cell clones dual-stained for both CD4 and CD8 (top row). Background irrelevant staining (FITC-conjugated mouse IgG) for the same clones is illustrated in the bottom row. The results are representative of two independent experiments yielding similar results.

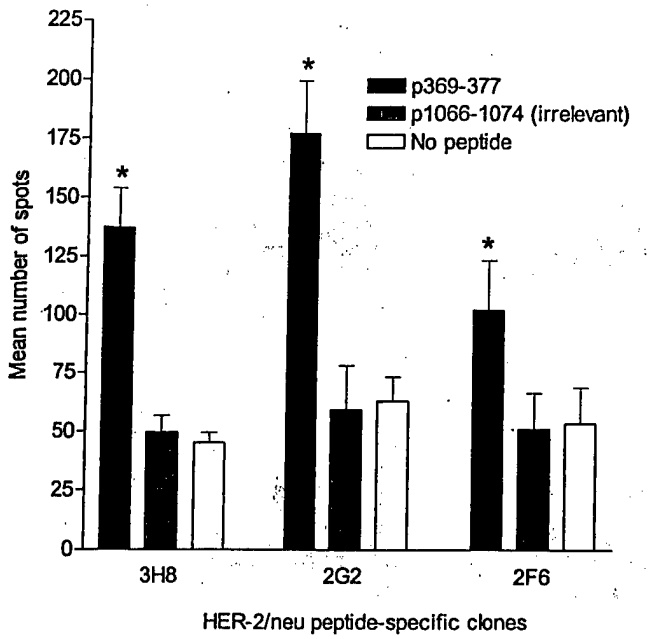

FIGURE 4 HER-2/neu peptide-specific αβ-TCR clones secrete IFN-γ in response to antigen. ELIspot data from αβ T-cell receptor (αβ-TCR) clones 3H8, 2G2, and 2F6 are depicted presented as the mean (± SEM; * = $p < 0.05$) number of spots calculated from quadruplicate determinations for clones stimulated with p369-377 (black bars), p1066-1074 (gray bars), or no peptide (white bars).

of spots detected in wells containing either no antigen (3H8, 45 ± 4, $p = 0.003$; 2G2, 63 ± 10, $p = 0.002$; 2F6, 54 ± 15, $p = 0.02$) or irrelevant peptide (3H8, 50 ± 7, $p = 0.03$; 2G2, 59 ± 19, $p = 0.03$; 2F6, 51 ± 15, $p = 0.05$). These clones also all released IFN-γ into the supernatant as assessed by ELISA (data not shown).

HER-2/neu peptide-specific αβ-TCR clones lyse HLA-$A2^+$, HER-2/neu^+ tumor cells. The clones were examined for lysis of peptide-loaded autologous BLCL or HER-2/neu-expressing tumor cell lines. As an example, two $CD3^+CD8^+CD4^-$ clones (2G2 and 2G7) and one $CD3^+CD8^-CD4^+$ (3G9) are revealed in Figure 5. The clones examined all demonstrated detectable lysis of p369-377-loaded BLCL and HLA-$A2^+$ HER-2/neu-expressing tumor cells. At an effector:target (E:T) ratio of 40:1, the percent of specific lysis was 9%, 12%, and 13% for clones 2G2 (Panel A), 2G7 (Panel B), and 3G9 (Panel C), respectively against p369-377-loaded autologous BLCL. Responses against non peptide-loaded BLCL were < 4% for all clones tested. Against the HLA-A2+, HER-2/neu-expressing SKOV3 tumor cell line, the percent of specific lysis at 40:1 E:T was 7%, 7%, and 6% for 2G2 (Panel A), 2G7 (Panel B), and 3G9 (Panel C), respectively. Lysis of parental, HER-2-expressing SKOV3 tumor cells was not detectable for any of the tested clones.

The γδ-TCR clones secrete IFN-γ and lyse HLA-$A2^+$, HER-2/neu-expressing tumor cells. The γδ-TCR clones were examined for expression of CD4, CD8 (Figure 6). Clone 3F7 was predominantly $CD8^+$. In contrast, clone 1D1 displayed heterogeneous but consistent expression of CD4 and CD8. As assessed by flow cytometry expression of the γδ-TCR was observed in 90% of cells (Figure 2). The remaining 10% expressed neither CD3 (not shown) nor γδ-TCR suggesting nonspecific particulate debris. Approximately 50% of 1D1 expressed CD8, 10% expressed CD4, and the remaining 40% did not express either CD8 or CD4 (Figure 6). Peptide-specific release of IFN-γ against p369-377, an irrelevant HLA-A2 HER-2/neu peptide p1066-1074, or no antigen was examined in an ELIspot assay (Figure 7). The mean number of spots detected in wells containing p369-377 was 57 (± 7, SEM) and 6 (± 1, SEM) for 1D1 and 3F7 respectively. The mean number of spots, in the presence of p369-377, for 1D1 was significantly higher than the mean number of spots detected in wells containing either no antigen (19 ± 4, SEM, $p = 0.015$) or irrelevant peptide (26 ± 8, SEM, $p = 0.03$). Clone 3F7 did not demonstrate significantly elevated IFN-γ release in response to peptide.

The γδ-TCR clones were also examined for lysis of HER-2/neu-expressing cells lines (Figure 8). Both clones displayed lysis of the HLA-$A2^+$, HER-2/neu-expressing tumor cell line SKOV3-A2. At an E:T ratio of 40:1, the percent of specific lysis was 25% and 20% for 1D1 (Panel A) and 3F7 (Panel B), respectively. Lysis of parental HER-2-expressing SKOV3 tumor cells was < 1% for both clones at all E:T ratios examined.

CONCLUSIONS

We immunized patients with T-helper epitopes derived from HER-2/neu, each of which encompassed known HLA class I motifs to generate HER-2/neu-specific CTL in cancer patients [8]. Clinical vaccination with longer peptides encompassing HLA class I binding motifs allowed exogenous uptake of helper epitopes within the HLA class II processing pathway. Once internalized and processed, peptides are available to the HLA-class I processing pathway for presentation [16]. Thus, the subsequent stimulation of HLA class I restricted T cells represents an immune response that likely required internal processing and presentation of antigen rather than exogenous peptide loading. We cloned T cells from a patient successfully immunized with a T-helper peptide (p369-386) derived from HER-2/neu to evaluate the repertoire of the HLA class I specific peptide (p369-377) encompassed within the longer epitope. The studies described here demonstrate substantial diversity of the vaccinated repertoire, which consisted not only of $CD4^+$ and $CD8^+$

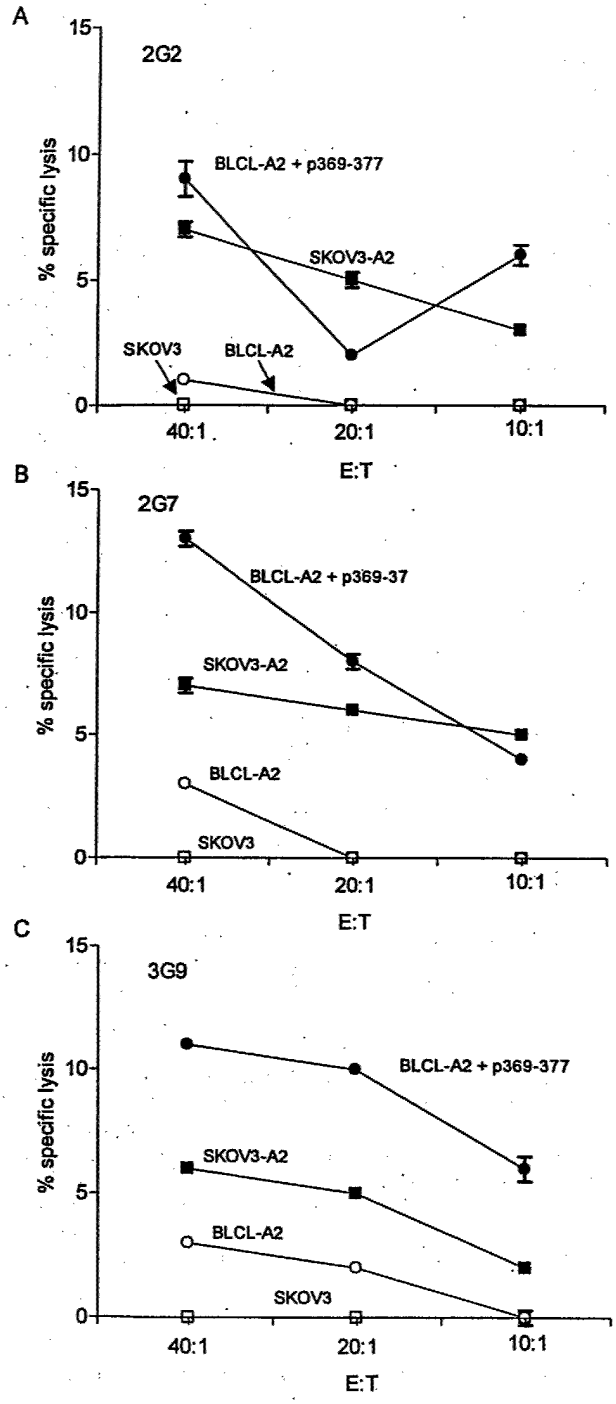

FIGURE 5 HER-2/neu peptide-specific αβ T-cell receptor (αβ-TCR) clones lyse human leukocyte antigen A2 (HLA-$A2^+$), HER2/neu-expressing tumor cells. Cytolytic activity data from αβ-TCR clones 2G2 (Panel A), 2G7 (Panel B), and 3G9 (Panel C) are depicted against BLCL-A2 alone (open circles), p369-377-loaded BLCL-A2 (closed circles), or the HER-2/neu-overexpressing tumor cells SKOV3 (open squares) and SKOV3-A2 (closed squares). The data are presented as the mean (± SEM) of triplicate determinations at each of three E:T ratios, 40:1, 20:1, and 10:1. The absence of a standard error bar indicates a SEM of < 0.5% specific lysis.

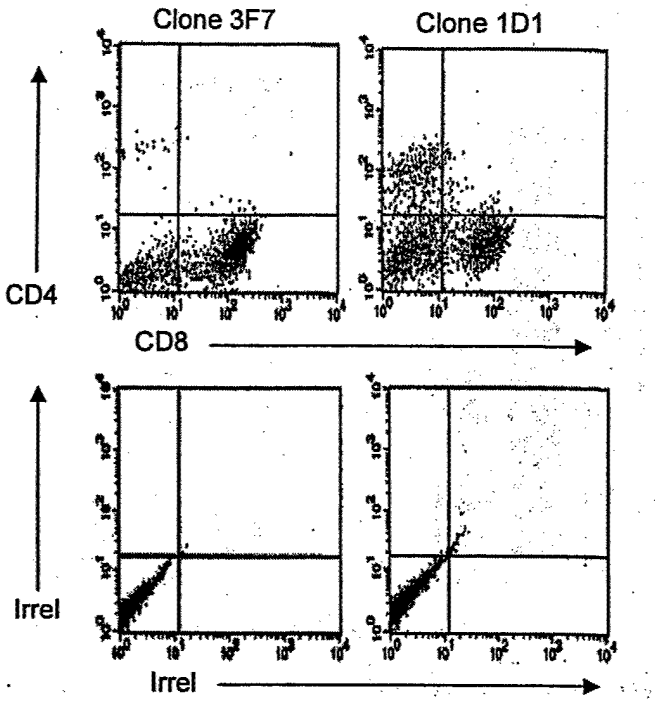

FIGURE 6 The γδ T-cell receptor (γδ-TCR) T-cell clones could be isolated following HER-2/neu vaccination. This illustrates the flow cytometry data of p369-377-specific γδ-TCR T-cell clones dual-stained for both CD4 and CD8 (top row). Background irrelevant staining (FITC-conjugated anti-mouse IgG) for the same clones are depicted in the bottom row. The results are representative of two independent experiments yielding similar results.

T cells, but also αβ-TCR and γδ-TCR T cells specific for HLA class I, HER-2/neu peptide antigen.

As expected, the majority of peptide-specific clones isolated were $CD8^+$; however, nearly 25% of the clones identified were $CD4^+$. A representative clone of the $CD4^+$ T-cell population could lyse both peptide-loaded BLCL as well as HER-2/neu overexpressing HLA-A2 tumor cells. The lysis observed by $CD4^+$ T-cell clones was weak and of similar magnitude to the lysis observed by the $CD8^+$ T-cell clones, a finding that is consistent with previous studies of HER-2/neu-specific T-cell lines or clones [4, 10, 15, 17]. Isolation of T cells with weak lytic activity is likely due to loss of higher affinity T cells against self antigens by tolerizing mechanisms [18]. HLA class II restricted cytolytic $CD4^+$ T cells have been defined and have been described to play a roles in the pathogenesis of autoimmune disease via responses to self antigens, such as myelin [19]. Reports of HLA class I restricted CD4+ T cells are rare. There have been only a few reports of cytolytic $CD4^+$ T cells responding to HLA class I peptides in melanoma [17, 20]. One study was a report of a HLA-B57-restricted $CD4^+$ cytolytic clone, which could lyse autologous melanoma and allogeneic

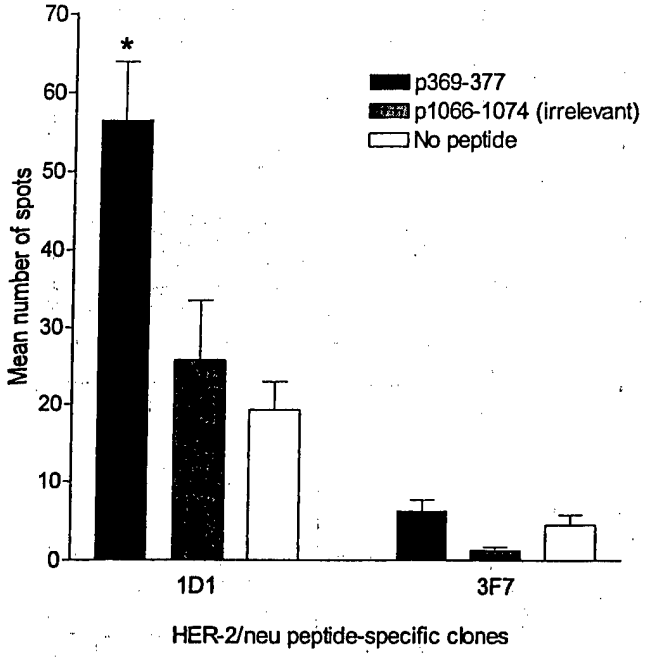

FIGURE 7 One of the $CD8^+$ γδ T-cell receptor (γδ-TCR) clones, 1D1 secretes IFN-γ in response to antigen. ELIspot data from γδ-TCR clones 1D1 and 3F7 are depicted and presented as the mean (± SEM; * = $p < 0.05$) number of spots calculated from quadruplicate determinations for clones stimulated with p369-377 (black bars), p1066-1074 (gray bars), or no peptide (white bars).

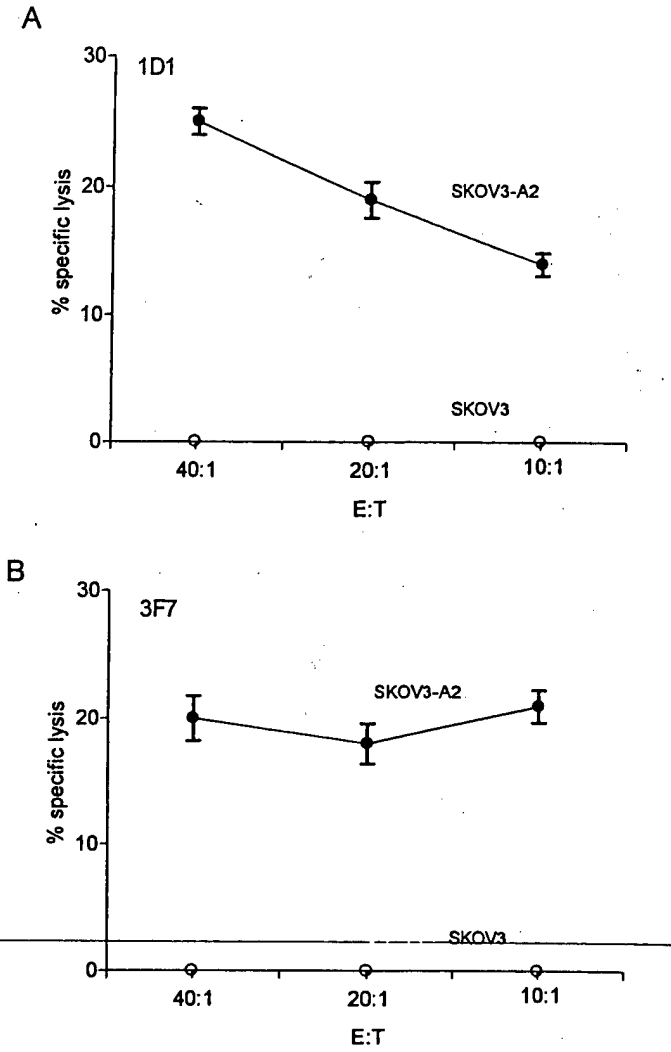

FIGURE 8 The CD8′2b γδ T-cell receptor (γδ-TCR) clones displayed human leukocyte antigen A2 (HLA-A2) restricted, $HER2^+$ tumor cell lysis. Cytolytic activity data from γδ-TCR clones 1D1 (Panel A) and 3F7 (Panel B) are depicted against the HER-2/neu overexpressing tumor cells SKOV3 (open circles) and SKOV3-A2 (closed circles). The data are presented as the mean (± SEM) of triplicate determinations at each of three E:T ratios, 40:1, 20:1, and 10:1.

melanoma lines that were matched for HLA-B57 [16]. Nishimura and colleagues [17] performed a more extensive analysis of a $CD4^+$ T-cell line derived from tumor infiltrating lymphocytes derived from a melanoma patient. The $CD4^+$ T cells were specific for a tyrosinase peptide, p368-376, secreted cytokines of a Th1 phenotype and were weakly cytolytic. Furthermore, although the cell line demonstrated high CD4 expression, CD4 apparently was not involved in antigen-specific signaling through the TCR. The authors concluded that the HLA class I restricted $CD4^+$ T cell was most likely a rare event with unknown functional significance. Data presented here suggests the HLA class I restricted $CD4^+$ T cells may be present in number greater than previously thought. Whether the functional importance of these cells lay in direct killing or regulating the immune response through cytokine secretion is currently unknown.

Most analyses of the T-cell repertoire generated in response to dominant HLA class I epitopes have focused exclusively αβ-TCR^+, $CD3^+$, $CD8^+$ T cells. Specifically, investigations have analyzed the Vβ usage and the CDR3 size of the αβ-TCR of viral peptide-specific T cells [21, 22]. In general, findings from viral studies suggest that the repertoire to dominant viral epitopes is tightly restricted in terms of TCR usage. Recently, however, Dietrich and colleagues [23] examined the clonality of the T-cell repertoire to an HLA-A2 restricted immunodominant epitope of the melanoma-associated antigen, Melan-A. They observed, by analyzing the Vβ segment and CDR3 lengths, that the natural repertoire to this self peptide was considerably broader than what had been previously observed in viral peptide systems, suggesting that the αβ-TCR usage is not as narrowly restricted in response to tumors as it is in viral infections. As expected, in the present study, the predominant TCR was the αβ-TCR. However, 2 of 21 clones (10%) expressed

γδ-TCR. γδ-TCR T cells are involved in a wide range of immune responses to infectious and non-infectious diseases, including malaria, mycobacterial infections, cancers, and autoimmune disorders, such as multiple sclerosis [24]. The γδ-TCR T-cell clones have been isolated from tumor-infiltrating lymphocytes derived from tumors, such as dysgerminoma [25], seminoma [25], renal carcinoma [26], lung [27], colorectal [28], and melanoma [29]. Tumor-associated γδ-TCR T cells that have been described in colorectal cancers have a $CD8^+$, cytolytic phenotype similar to those described in the present study [27]. In the present study, γδ-TCR T-cell clone 1D1, displayed heterogeneous expression of CD4 or CD8. In general, circulating γδ-TCR T cells do not express CD4 or CD8, which is distinct from αβ-TCR T cells in which either CD4 or CD8 expression occurs during early thymocyte ontogeny [30]. Little is known about the regulation of expression of CD4 and CD8 in γδ-TCR T cells, but since the majority of circulating γδ-TCR T cells are $CD4^-/CD8^-$, it is thought that some coreceptor upregulation may be due to environmental maturation [24, 31]. Therefore, it is possible that the differential expression of CD8 and CD4 in our clonal population was induced by *in vitro* maturation that could have led to clonal divergence [32].

In autoimmune diseases, pathology has been attributed to infiltrating γδ-TCR T cells. For example, antibody depletion of γδ-TCR reduces demyelination and inflammation in experimental murine multiple sclerosis [33]. It is unknown if autoreactive γδ-TCR T cells respond secondarily to damaged and stressed tissue [34] or if they initiate autoimmunity directly. One hypothesis [35] is that, given the broad range of regulation by multiple mechanisms of antigen presentation and natural localization to epithelial tissue, γδ-TCR T cells are sentinels for the immune system and are capable of alerting the immune system to the presence of danger (*e.g.*, infection, tumors, etc.).

Immunization against tumor antigens in cancer patients with peptide-based vaccines has been best studied in patients with existing disease burden and clinical responses have occurred only infrequently [36, 37]. We hypothesize that vaccines may be better suited for the prevention of relapse following conventional therapies [8]. Nevertheless, characterization of the T-cell response to tumor antigen-specific peptide vaccines is necessary for optimizing design and improving clinical outcome. The results presented here demonstrate that the T-cell population specific for the HER-2/neu peptide, p369-377 elicited after active immunization is polyclonal both at the level of the TCR and the T-cell subsets stimulated. These data reflect the distinct differences between the highly monoclonal immunodominant responses directed against foreign infectious disease antigens and the diverse polyclonal response against self tumor antigens. Many of the elements in the immune response described in this report are important in the autoimmunity (*i.e.*, $CD4^+$ cytolytic T cells and γδTCR T cells). Perhaps functional immunity directed against specific self antigens mimics the pathogenic pathways of autoimmune disease closer than anticipated.

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REFERENCES

1. Eisenbach L, Bar-Haim E, El-Shami K: Antitumor vaccination using peptide based vaccines. Immunol Lett 74:27, 2000.
2. Disis ML, Gralow JR, Bernhard H, Hand SL, Rubin WD, Cheever MA: Peptide-based, but not whole protein, vaccines elicit immunity to HER-2/neu, oncogenic self-protein. J Immunol 156:3151, 1996.
3. Hunziker IP, Zurbriggen R, Glueck R, Engler OB, Reichen J, Dai WJ, Pichler WJ, Cerny A: Perspectives: towards a peptide-based vaccine against hepatitis C virus. Mol Immunol 38:475, 2000.
4. Zaks TZ, Rosenberg SA: Immunization with a peptide epitope (p369-377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu+ tumors. Cancer Res 58:4902, 1998.
5. Chen W, Norbury CC, Cho Y, Yewdell JW, Bennink JR: Immunoproteasomes shape immunodominance hierarchies of antiviral CD8(+) T cells at the levels of T cell repertoire and presentation of viral antigens. J Exp Med 193:1319, 2001.
6. Irvine KR, McCabe BJ, Rosenberg SA, Restifo NP: Synthetic oligonucleotide expressed by a recombinant vaccinia virus elicits therapeutic CTL. J Immunol 154:4651, 1995.
7. Bonini C, Lee SP, Riddell SR, Greenberg PD: Targeting antigen in mature dendritic cells for simultaneous stimulation of CD4(+) and CD8(+) T cells. J Immunol 166:5250, 2001.
8. Knutson KL, Schiffman K, Disis ML: Immunization with a HER-2/neu helper peptide vaccine generates of HER-2/neu CD8 T cell immunity in cancer patients. J Clin Invest 107:477, 2001.

9. Fisk B, Hudson JM, Kavanagh J, Wharton JT, Murray JL, Ioannides CG, Kudelka AP: Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patients to HER-2 peptides. Anticancer Res 17:45, 1997.
10. Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, Eberlein TJ: Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc Natl Acad Sci U S A 92:432, 1995.
11. Ioannides CG, Fisk B, Fan D, Biddison WE, Wharton JT, O'Brian CA: Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptide derived from the HER-2/neu proto-oncogene. Cell Immunol 151:225, 1993.
12. Disis ML, Grabstein KH, Sleath PR, Cheever MA: Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. Clin Cancer Res 5:1289, 1999.
13. Disis ML, Bernhard H, Gralow JR, Hand SL, Emery SR, Calenoff E, Cheever MA: Immunity to the HER-2/neu oncogenic protein. Ciba Found Symp 187:198, 1994.
14. Reece JC, Geysen HM, Rodda SJ: Mapping the major human T helper epitopes of tetanus toxin. The emerging picture. J Immunol 151:6175, 1993.
15. Fisk B, Blevins TL, Wharton JT, Ioannides CG: Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J Exp Med 181:2109, 1995.
16. Rock KL, Clark K: Analysis of the role of MHC class II presentation in the stimulation of cytotoxic T lymphocytes by antigens targeted into the exogenous antigen-MHC class I presentation pathway. J Immunol 156:3721, 1996.
17. Nishimura MI, Avichezer D, Custer MC, Lee CS, Chen C, Parkhurst MR, Diamond RA, Robbins PF, Schwartzentruber DJ, Rosenberg SA: MHC class I-restricted recognition of a melanoma antigen by a human CD4+ tumor infiltrating lymphocyte. Cancer Res 59:6230, 1999.
18. Slansky JE, Rattis FM, Boyd LF, Fahmy T, Jaffee EM, Schneck JP, Margulies DH, Pardoll DM: Enhanced antigen-specific antitumor immunity with altered peptide ligands that stabilize the MHC-peptide-TCR complex. Immunity 13:529, 2000.
19. Martino G, Hartung HP: Immunopathogenesis of multiple sclerosis: the role of T cells. Curr Opin Neurol 12:309, 1999.
20. Somasundaram R, Robbins P, Moonka D, Loh E, Marincola F, Patel A, Guerry D, Herlyn D: CD4(+), HLA class I-restricted, cytolytic T-lymphocyte clone against primary malignant melanoma cells. Int J Cancer 85:253, 2000.
21. Lim DG, Bieganowska BK, Freeman GJ, Hafler DA: Examination of CD8+ T cell function in humans using MHC class I tetramers: similar cytotoxicity but variable proliferation and cytokine production among different clonal CD8+ T cells specific to a single viral epitope. J Immunol 165:6214, 2000.
22. Chen ZW, Li Y, Zeng X, Kuroda MJ, Schmitz JE, Shen Y, Lai X, Shen L, Letvin NL: The TCR repertoire of an immunodominant CD8(+) T lymphocyte population. J Immunol 166:4525, 2001.
23. Dietrich PY, Walker PR, Quiquerez AL, Perrin G, Dutoit V, Lienard D, Guillaume P, Cerottini JC, Romero P, Valmori D: Melanoma patients respond to a cytotoxic T lymphocyte-defined self-peptide with diverse and non-overlapping T-cell receptor repertoires. Cancer Res 61: 2047, 2001.
24. Boismenu R, Havran WL: Gammadelta T cells in host defense and epithelial cell biology. Clin Immunol Immunopathol 86:121, 1998.
25. Zhao X, Wei YQ, Kariya Y, Teshigawara K, Uchida A: Accumulation of gamma/delta T cells in human dysgerminoma and seminoma: roles in autologous tumor killing and granuloma formation. Immunol Invest 24:607, 1995.
26. Choudhary A, Davodeau F, Moreau A, Peyrat MA, Bonneville M, Jotereau F: Selective lysis of autologous tumor cells by recurrent gamma delta tumor-infiltrating lymphocytes from renal carcinoma. J Immunol 154:3932, 1995.
27. Yu S, He W, Chen J, Zhang F, Ba D: Expansion and immunological study of human tumor infiltrating gamma/delta T lymphocytes in vitro. Int Arch Allergy Immunol 119:31, 1999.
28. Watanabe N, Hizuta A, Tanaka N, Orita K: Localization of T cell receptor (TCR)-gamma delta + T cells into human colorectal cancer: flow cytometric analysis of TCR-gamma delta expression in tumour-infiltrating lymphocytes. Clin Exp Immunol 102:167, 1995.
29. Bachelez H, Flageul B, Degos L, Boumsell L, Bensussan A: TCR gamma delta bearing T lymphocytes infiltrating human primary cutaneous melanomas. J Invest Dermatol 98:369, 1992.
30. Ellmeier W, Sawada S, Littman DR: The regulation of CD4 and CD8 coreceptor gene expression during T cell development. Annu Rev Immunol 17:523, 1999.
31. Kabelitz D, Wesch D, Hinz T: gamma delta T cells, their T cell receptor usage and role in human diseases. Springer Semin Immunopathol 21:55, 1999.
32. Goodman T, Lefrancois L: Expression of the gamma-delta T-cell receptor on intestinal CD8+ intraepithelial lymphocytes. Nature 333:855, 1988.
33. Rajan AJ, Gao YL, Raine CS, Brosnan CF: A pathogenic role for gamma delta T cells in relapsing-remitting experimental allergic encephalomyelitis in the SJL mouse. J Immunol 157:941, 1996.
34. Hayday A, Geng L: Gamma delta cells regulate autoimmunity. Curr Opin Immunol 9:884, 1997.
35. De Libero G: Tissue distribution, antigen specificity and effector functions of gamma delta T cells in human diseases. Springer Semin Immunopathol 22:219, 2000.
36. Gjertsen MK, Buanes T, Rosseland AR, Bakka A, Glad-

haug I, Soreide O, Eriksen JA, Moller M, Baksaas I, Lothe RA, Saeterdal I, Gaudernack G: Intradermal ras peptide vaccination with granulocyte-macrophage colony-stimulating factor as adjuvant: clinical and immunological responses in patients with pancreatic adenocarcinoma. Int J Cancer 92:441, 2001.

37. Lee KH, Wang E, Nielsen MB, Wunderlich J, Migueles S, Connors M, Steinberg SM, Rosenberg SA, Marincola FM: Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to in vitro stimulation but does not lead to tumor regression. J Immunol 163:6292, 1999.

Immunization of cancer patients with HER-2/neu derived peptides demonstrating high affinity binding to multiple class II alleles.

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Abbreviations:

APC: antigen presenting cell

CEA: carcinoembryonic antigen

CTL: cytotoxic T lymphocyte

DTH: delayed type hypersensitivity

ECD: extracellular domain of HER-2/neu

HLA: human leukocyte antigen

ICD: intracellular domain of HER-2/neu

IFA: incomplete Freund's adjuvant

MHC: major histocompatibility complex

PADRE: pan DR epitope

PBMC: peripheral blood mononuclear cells

SI: stimulation index

TCR: T cell receptor

ABSTRACT

Purpose: The purpose of this study was to immunize patients with HER-2/neu overexpressing cancer with a multi-peptide vaccine comprised of four class II HER-2/neu peptides that had been identified as the most immunogenic in a previous clinical trial. Furthermore, we questioned if MHC binding affinity could predict the *in vivo* immunogenicity of the HER-2/neu helper peptides.

Experimental Design: Four putative class II HER-2/neu peptides which were found to generate detectable specific T cell responses (S.I. > 2) in a majority of patients in a previous study were used to formulate a single vaccine. The multi-peptide vaccine was administered intradermally with GM-CSF as an adjuvant. Ten patients with HER-2/neu overexpressing breast or lung cancer were enrolled. HER-2/neu peptide-and protein-specific T cell and antibody immune responses were measured. Competitive inhibition assays were used to analyze the class II HER-2/neu peptides for their binding affinity to 14 common HLA-DR alleles.

Results: Twenty-five percent of patients developed HER-2/neu peptide-specific T cell immunity and 50% developed HER-2/neu peptide-specific antibody immunity. No patient developed HER-2/neu protein-specific T cell or antibody immunity. The majority of peptides exhibited high binding affinity, *in vitro,* to three or more of the 14 DR alleles analyzed.

Conclusion: The group of peptides used in this study demonstrated high binding affinity to multiple DR alleles suggesting that *in vitro* binding affinity may be able to predict the *in vivo* immunogenicity of class II peptides. However, only a minority of patients immunized with the multi-peptide vaccine developed HER-2/neu peptide-specific T cell or antibody immunity and none developed HER-2/neu protein-specific immunity.

INTRODUCTION

Studies have now shown that tumor antigen specific peptide-based vaccines are effective in generating immune responses to self-proteins (1, 2). Furthermore, the inclusion of well-defined MHC class II epitopes in tumor antigen specific vaccines, appear to play an important role in augmenting the immune response. A current focus of study is identifying immunogenic MHC class II epitopes of self-tumor antigens for use in peptide vaccines. In addition, methods of delivering multiple epitopes, including multi-peptide vaccines are being developed. Over the last several years the identification of MHC class II binding epitopes to common tumor antigens has been facilitated by improvements in the ability to predict MHC class II binding. More specifically, methods utilizing quantitative binding assays which associate *in vitro* high peptide-DR binding affinity with immunogenicity of class II epitopes (3) have been developed but have yet to be demonstrated *in vivo*.

As a result of an earlier HER-2/neu peptide vaccine study incorporating putative HER-2/neu T-helper epitopes (1), we questioned whether formulating a peptide vaccine comprised of multiple immunogenic class II peptides would result in a more robust immune response. Thus, in this study we evaluated whether active immunization with a multi-peptide vaccine comprised of four HER-2/neu T helper epitopes which had been identified as the most immunogenic in a previous study, would generate HER-2/neu peptide- and protein-specific immunity. Furthermore, we questioned if *in vitro* DR binding affinity could predict the *in vivo* immunogenicity of these antigenic HER-2/neu helper peptides

METHODS AND MATERIALS

Patient population. The University of Washington Human Subjects Division and the United States Food and Drug Administration approved a Phase I trial of a HER-2/neu multi-peptide vaccine. Ten patients with HER-2/neu overexpressing Stage III (n=5) or IV (n=4) breast, and stage III non-small cell lung cancer (n=1) gave informed consent and were enrolled in the study according to institutional and federal regulations. Median age was 54 (range 40-69) years and median time from last chemotherapy was 10 (range 8-16) months. Criteria for entry to the study were identical to previously described vaccine trials (1). Four peptides derived from the HER-2/neu protein sequence were formulated into a single vaccine and admixed with 125 μg. GM-CSF (Immunex Corporation, Seattle, WA). Vaccines were administered intradermally once monthly for 6 months to the same regional draining lymph node site. Toxicity was graded according to NCI Common Toxicity Scoring defined prior to August 1998 (4). Ten patients met eligibility criteria and were enrolled on the study, 9 of whom completed all 6 vaccines. Two patients did not complete the study, 1 due to progressive disease requiring treatment during the vaccine series. The other patient dropped out of the study for personal reasons prior to completing scheduled immunologic follow-up after completing the vaccine series. Immune response data presented here details the 8 patients who completed all 6 vaccines and scheduled immunologic follow-up.

HER-2/neu multi-peptide based vaccine. Four peptides derived from the HER-2/neu protein sequence were identified as being the most immunogenic after analysis of a previously performed Phase I study in which nine HER-2/neu peptides were used to formulate three peptide-based vaccines (1). The four peptides were determined to be the most immunogenic based on the ability to generate a detectable immune response after active immunization in the greatest number of patients (Table 1). The peptides

were constructed and formulated as previously described (1). The four HER-2/neu peptides included in the current vaccine formulation were; p98-114 (p98), p369-384 (p369) derived from the extracellular domain (ECD) of the HER-2/neu protein and p776-790 (p776) and p927-941 (p927) derived from the intracellular domain (ICD) of the HER-2/neu protein (Corixa Corp., Seattle, WA). The peptide dose in each vaccination was 500 μg/peptide.

HER-2/neu peptide binding affinity for Class II MHC. Binding of HER-2/neu derived peptides to HLA-DR molecules was tested using competitive inhibition assays performed as previously described (3). Briefly, purified human class II molecules, 5 to 500 nM, an excess of ^{125}I-radiolabeled probe peptides, and various doses of each unlabeled peptide were co-incubated for 48 hours in the presence of a protease inhibitor cocktail. Following the incubation period, MHC-peptide complexes were separated from unbound radiolabeled peptide by one of two methods: size-exclusion gel-filtration chromatography, or capture of complexes utilizing anti-DR mAb LB3.1. The percent of bound radioactivity was then determined. The concentration of unlabeled peptide required to inhibit the binding of the labeled peptide by 50% (IC_{50}) was determined by plotting dose versus % inhibition. Under conditions where [label] < [MHC] and $IC_{50} \geq$ [MHC], the measured IC_{50} values are reasonable approximations of true K_d values. Significant binding affinity was defined as 1500 nM.

Detection of peripheral blood T cell responses. T cell proliferation was assessed using a modified limiting dilution assay designed for detecting low frequency lymphocyte precursors based on Poisson distribution (5) and as previously described (6). Results are reported as a standard stimulation index (SI), defined as the mean of all 24 experimental wells divided by the mean of the control wells (no antigen). Phytohemagglutinin incubated with patient T cells at a concentration of 5 μg/ml, was used as

a positive control for the ability of T cells to respond to antigen and resulted in an S.I. > 2.0 in all assays reported (data not shown). Peripheral blood mononuclear cells (PBMC) from 30 female volunteer donors without cancer, age range 32-58, were evaluated in similar assays to establish baseline values. The mean and 3 standard deviations of the T cell response in the reference population to any of the HER-2/neu antigens tested was a maximum S.I. of 1.98, therefore an S.I. > 2 was considered evidence of an immunized response. If subjects had a S.I. > 2 at baseline, i.e. pre-existent immunity to HER-2/neu (7), a post-vaccination response was defined as positive if it was a minimum of 2 times baseline.

Determination of HER-2/neu peptide antibodies. 96-well microtiter plates (Dynex Technologies, Inc., Chantilly, VA), were coated with HER-2/neu peptides at a concentration of 20 μg/ml, diluted with carbonate buffer and added at 50 μl per well alternating with wells coated with 50 μl/well of carbonate buffer alone. One row consisted of purified IgG (Sigma Chemical Co., St. Louis, MO) to generate a standard curve. After overnight incubation, all wells were blocked with 1% casein/PBS, 100 μl/well and incubated at room temperature for 1-2 hours. Plates were then washed with a 0.15% casein/1% PBS/0.05% Tween-20 wash buffer 4 times before experimental serum diluted in 10% FCS/PBS/1% BSA/25 μg/ml mouse IgG was added at 1:100, 1:200, 1: 400 and 1:800 dilutions. Plates were incubated for 2 hours at room temperature. Plates were then washed 4 times with casein-based wash and incubated for 45 minutes at room temperature after addition of 50 μl/well IgG-HRP conjugate (Zymed Laboratories, San Francisco, CA) diluted 1:10,000 in PBS/BSA buffer. After a final 4 washes with casein-based wash buffer, TMB reagent (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added 75 μl/well and color reaction read at 640 nm until the well containing the 0.16 μg/ml standard reached an OD of 0.3. Reactions were stopped with 75 μl/well 1N HCL and read at 450 nm.

The OD of each serum dilution was calculated as the OD of the peptide-coated wells minus the OD of the buffer-coated wells. Values for μg/ml were calculated from the log-log equation of the line for the standard curve on each plate. A positive sample was defined as an antibody concentration greater than the mean of a volunteer blood donor population and 3 standard deviations, 0.07+/-0.26 μg/ml (n=108). Some patients did have pre-existent detectable antibody immunity to an immunizing peptide and were only considered to have responses if they boosted the antibody level to twice their baseline value. The accuracy of the HER-2/neu peptide antibody assay was described with an average coefficient of variation (CV) of 12%, linearity by a correlation coefficient of 0.97. Based on repeated measures of 20 volunteer and 20 experimental sera over a 6-month period the intra and inter-assay coefficient of variations were 12% and 18%, respectively.

Determination of HER-2/neu protein antibodies. Analyses were performed as previously described (7). Serum from a patient with a documented HER-2/neu specific antibody response was used as a positive control (8). A positive sample was defined as an antibody concentration greater than the mean of a volunteer blood donor population and 2 standard deviations, 0.20+/-0.4 μg/ml (n=200). Positive results were confirmed by Western blot analysis. Some patients did have pre-existent detectable antibody immunity to the HER-2/neu protein and were only considered to have responses if they boosted the antibody level to twice their baseline value. The specificity of the assay was 78% and sensitivity 90%. The accuracy of the HER-2/neu protein antibody assay was described with an average CV of 10%, linearity by a correlation coefficient of 0.99. Based on repeated measures of 20 volunteer and 20 experimental sera over a 6-month period the intra and inter-assay coefficient of variations were 15% and 9%, respectively.

Statistical methods. To determine whether the percentage of patients who developed an immune response after active immunization correlated with the number of DR alleles with significant binding for specific alleles, Pearson's correlation coefficient was estimated. The data used for this analysis were simply the percentage of patients with a response for a particular peptide and the corresponding percentage of alleles with significant binding. No adjustment was made to account for the fact that the number of patients immunized with a particular peptide varied, as this number was relatively constant (11 to 14).

RESULTS

Immunodominant HER-2/neu peptide epitopes determined by in vivo immunogenicity also bind MHC class II molecules with high affinity. Nine peptides used in active immunization in a previously reported study (1), were analyzed for their binding affinity to 14 HLA-DR alleles (Table 2). Of the nine peptides evaluated p98, p369, p776 and p927 were considered the most immunogenic *in vivo* and formulated into a single vaccine used in the current study (Table 1). All 14 DR alleles tested were shown to have high *in vitro* binding affinity, defined as 1500 nM, to at least one of the nine HER-2/neu peptides originally assessed (Table 2). Five of 9 peptides had high binding affinity, *in vitro,* to three or more of the 14 DR alleles analyzed. p328 and p927 were not associated with significant binding of any of the DR alleles evaluated.

The affinity of in vitro peptide binding correlates with in vivo immunogenicity. Assessment of binding of the HER-2/neu peptides to class II MHC molecules revealed that the percentage of high binding DR alleles for a given peptide significantly correlated with the percentage of patients who responded to that peptide when it was administered as a vaccine, R=0.76 (95% CI is 0.50 to 1.0) (Fig. 1).

Patients enrolled had MHC Class II alleles capable of binding HER-2/neu immunizing peptides. Three of the 4 immunogenic HER-2/neu peptides, p98, p369, and p776, used for vaccination had high *in vitro* binding affinity to 1 or more of the HLA-DR alleles analyzed (Table 2). All 8 patients evaluated had 2 or more DR alleles associated with high binding affinity of HER-2/neu peptides in their immunizing mix (Table 3).

The minority of patients immunized with the vaccine containing peptides associated with high affinity binding to multiple DR alleles developed HER-2/neu peptide-specific T cell immunity and none developed HER-2/neu protein-specific T cell immunity. Figure 2 shows the pre- and post-immunization T cell responses of the 8 patients who completed 6 vaccines. Two of 8 (25%) patients developed immune response to at least one of the HER-2/neu peptides, one to p369 (S.I 6.5) and one to p776 (S.I. 2.4). Four patients had pre-existent immune responses to one or more peptides, 3 patients to p98 (S.I. 2.0, 17.3 and 18.1), 1 patient to p369 (S.I. 3.9), 3 patients to p776 (S.I. 2.7, 2.8, and 2.9), and 3 patients to p927 (S.I. 2.8, 6.4, and 6.7). All patients who had pre-existent immune responses had a decrease in S.I. post-immunization. None of the patients developed HER-2/neu protein-specific immunity after peptide immunization (Fig. 2). Five patients had pre-existent immune responses to either ICD or ECD. All 5 patients had pre-existent immune responses to ICD (range S.I. 4.2-11.6) and 3 of the 5 patients responded to ECD prior to immunization (range S.I. 3.2-3.5). All 5 patients with pre-existent immunity had a decrease in S.I. post-immunization.

The vaccine was very well tolerated with only 5 events of grade 1 toxicities that included headache, hematuria, chills, and, localized infection at the vaccination site in 4 patients. No patient developed any detectable evidence of autoimmune toxicity particularly in organs known to express basal levels of HER-2/neu protein such as liver, digestive tract, and skin (9).

The minority of patients immunized with the vaccine containing peptides associated with high affinity binding to multiple DR alleles vaccine developed HER-2/neu peptide-specific antibody immunity and none developed HER-2/neu protein-specific antibody immunity. Figure 3 demonstrates the pre- and post-immunization peptide- and protein-specific antibody responses of the 8 patients who completed 6

vaccines. Four patients developed antibodies to one or more HER-2/neu peptides in the peptide mix, 2 patients to p98 (1.1 and 2.07 μg/ml), 1 patient to p776 (4.6 μg/ml), and 2 patients to p927 (1.6 and 2.3 μg/ml). None of the patients developed HER-2/neu protein-specific antibodies post-immunization. Only a few patients had pre-existent antibody immune responses to either peptides or protein, 1 patient to p98 (0.93 μg/ml) and 2 patients to protein (1.5 and 6.3 μg/ml).

DISCUSSION

The focus of this study was to immunize patients with HER-2/neu overexpressing cancers with a multi-peptide vaccine comprised of four HER-2/neu peptides that had been identified as the most immunogenic, i.e. those that generated immunity in the majority of patients immunized in a previous study (1). Further analysis demonstrated that these four HER-2/neu peptides were associated with high affinity binding to multiple DR alleles. Thus, immunodominant HER-2/neu peptide epitopes determined by *in vivo* immunogenicity also bind MHC class II molecules with high affinity *in vitro*. However, only a minority of patients immunized with the multi-peptide vaccine developed HER-2/neu peptide-specific T cell or antibody immunity and none developed HER-2/neu protein-specific immunity.

The *in vivo* immunogenicity of HER-2/neu peptides could have been predicted by *in vitro* binding to MHC class II alleles. It is well known that MHC class I molecules recognize small peptide epitopes, 8-12 residues in size, which bind specific alleles (10). Binding affinity is critical to MHC class I molecules and the extent to which epitopes are able to bind determine the immunogenicity of the given epitope (11). In fact, modification of peptide motifs to increase binding results in improved immunogenicity (12). Investigators have shown that replacement of certain amino acids in the peptide sequences of CEA and gp100-derived epitopes result in enhanced MHC binding and induction of a greater number of antigen specific CTL (12, 13). Several algorithms have now been developed to predict peptide binding motifs for the most common MHC class I molecules. Unlike MHC class I, MHC class II molecules interact with larger peptides, are promiscuous and are able to bind multiple alleles (3, 14). *In vitro* binding studies have suggested that high affinity binding to MHC class II molecules is associated with immunogenicity of a given peptide epitope (3). Data presented here in

the present study demonstrates that binding is able to predict the immunogenicity of class II peptides. Interestingly, the peptide, p927, which was shown to be immunogenic *in vivo* demonstrated low affinity DR allele binding *in vitro*. This observation is not surprising as several studies investigating tolerance in autoimmune disease models have described epitopes displaying only negligible affinity for MHC class II molecules that occasionally become immunodominant in preference to other epitopes known to display far higher binding affinities (15). Although we made our vaccine based on the *in vivo* response from a previous study we could have used binding affinity to predict immunogenicity of peptides and predicted nearly the same vaccine formulation.

Unlike the parent study (1), few patients enrolled in this trial developed an immune response to HER-2/neu after active immunization with the four selected immunodominant HER-2/neu peptides. This current study utilized the same constructed peptides, as well as the same dose and route of administration as in the parent trial (1) so one would expect the vaccine to elicit peptide and protein specific immunity. The four peptides used were highly immunogenic *in vivo* and three of the four peptides had high binding affinity for several DR alleles *in vitro*. Furthermore, all the patients had HLA-DR alleles which were capable of binding one or more of the three high binding peptides. A possible explanation for the lack of observed immune response is potential competition of the immunodominant epitopes at the level of the MHC or T cell. MHC restricted epitopes have to compete with other peptides presumably at different levels and several factors such as enzymatic stability, uptake, intracellular transport, number of peptide molecules, flanking sequences, MHC binding, and T cell repertoire may influence the selection of immunogenic peptides (16). Competition at the level of the T cell may also occur and studies have shown that T cells directly compete with each other for antigen suggesting that epitope dominance may be the result of competitive interactions

between antigen bearing APC and T cells (17). Of note, however, studies in animal models using vaccines comprised of multiple class I epitopes have not supported lack of immunogenicity due to competition (18). Recent investigations using multi-epitope polypeptide vaccines comprised of high binding epitopes (9 CTL and PADRE epitopes) have been shown to induce both CD8+ IFN-γ and T-helper lymphocyte immune response (18).

Another potential reason for the lack of immune response after active immunization is theoretical immunosuppression in advanced stage disease cancer patients. Although the majority of patients in this study had advanced stage disease they were treated to a minimal or non-detectable disease state prior to enrolling in study. Furthermore, the patients in this study were similar to the cohort of patients in the previous study (1) in regards to age, disease burden and time from last chemotherapy. Lymphocyte PHA responses in this population did not differ significantly from normal donors (data not shown). Previous peptide vaccine studies by our group have demonstrated the ability to induce significant immune responses in heavily treated patients with minimal disease burden (1, 2).

Our goal was to determine the immunogenicity of a multi-peptide vaccine. Although predicted to be immunogenic by a previous study, the selected immunodominant peptides did not elicit immunity *in vivo* when formulated into a single vaccine. However, *in vitro* binding studies of the selected peptides did demonstrate high binding affinity which correlated with the development of immune response seen in the previous study. To our knowledge, this is one of the first studies to demonstrate that *in vitro* MHC binding affinity can predict the *in vivo* immunogenicity of MHC class II peptides. This correlation has significant implications in identifying immunogenic peptides when designing multi-

peptide vaccines. However, combination of immunodominant epitopes in a single vaccine may inhibit immunogenicity.

REFERENCES

1. Disis, M.L., Gooley, T.A., Rinn, K., Davis, D., Piepkorn, M., Cheever, M.A., Knutson, K.L., Schiffman, K. Generation of T-cell immunity to the her-2/neu protein after active immunization with her-2/neu Peptide-based vaccines. J Clin. Oncol., 20:2624-2632, 2002.

2. Knutson, K.L., Schiffman, K., Disis, M.L. Immunization with a HER-2/neu helper peptide vaccine generates HER- 2/neu CD8 T-cell immunity in cancer patients. J Clin. Invest., 107:477-484, 2001.

3. Southwood, S., Sidney, J., Kondo, A., del Guercio, M.F., Appella, E., Hoffman, S., Kubo, R.T., Chesnut, R.W., Grey, H.M., Sette, A. Several common HLA-DR types share largely overlapping peptide binding repertoires. J Immunol., 160:3363-3373, 1998.

4. NIH. Common Toxicity Scoring Criteria. National Institute of Health; http://ctep.info.nih.gov/CTC3, 1998.

5. Reece, J.C., Geysen, H.M., Rodda, S.J. Mapping the major human T helper epitopes of tetanus toxin. The emerging picture. J Immunol., 151:6175-6184, 1993.

6. Disis ML, Grabstein KH, Sleath PR, Cheever, M.A. Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. Clin. Cancer Res., 5:1289-1297, 1999.

7. Disis, M.L., Knutson, K.L., Schiffman, K., Rinn, K., McNeel, D.G. Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. Breast Cancer Res. Treat., 62:245-252, 2000.

8. Disis, M.L., Calenoff, E., McLaughlin, G., Murphy, A.E., Chen, W., Groner, B., Jeschke, M., Lydon, N., McGlynn, E., Livingston, R.B. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res., 54:16-20, 1994.

9. Press, M.F., Cordon-Cardo, C., Slamon, D.J. Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissues. Oncogene, 5:953-962, 1990.

10. Kawashima, I., Hudson, S.J., Tsai, V., Southwood, S., Takesako, K., Appella, E., Sette, A., Celis, E. The multi-epitope approach for immunotherapy for cancer: identification of several CTL epitopes from various tumor-associated antigens expressed on solid epithelial tumors. Hum. Immunol., 59:1-14, 1998.

11. Sette, A., Vitiello, A., Reherman, B., Fowler, P., Nayersina, R., Kast, W.M., Melief, C.J., Oseroff, C., Yuan, L., Ruppert, J. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. J. Immunol., 153:5586-5592, 1994.

12. Huarte, E., Sarobe, P., Lu, J., Casares, N., Lasarte, J.J., Dotor, J., Ruiz, M., Prieto, J., Celis, E., Borras-Cuesta, F. Enhancing immunogenicity of a CTL epitope from carcinoembryonic antigen by selective amino acid replacements. Clin. Cancer Res., 8:2336-2344, 2002.

13. Parkhurst, M.R., Salgaller, M.L., Southwood, S., Robbins, P.F., Sette, A., Rosenberg, S.A., Kawakami, Y. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. J. Immunol., 157:2539-2548, 1996.

14. Borras-Cuesta, F., Golvano, J., Garcia-Granero, M., Sarobe, P., Riezu-Boj, J., Huarte, E., Lasarte, J. Specific and general HLA-DR binding motifs: comparison of algorithms. Hum. Immunol., 61:266-278, 2000.

15. Fairchild, P.J., Wraith, D.C. Lowering the tone: mechanisms of immunodominance among epitopes with low affinity for MHC. Immunol. Today, 17:80-85, 1996.

16. Muller, C.P., Bunder, R., Mayser, H., Ammon, S., Weinmann, M., Brons, N.H.C., Scheider, F., Jung, G., Wiesmuller, K.H. Intramolecular immunodominance and intermolecular selection of

$H2^d$-restricted peptides define the same immunodominant region of the measles virus fusion protein. Mol. Immunol., 32:37-47, 1994.

17. Kedl, R.M., Rees, W.A., Hildeman, D.A., Schaefer, B., Mitchell, T., Kappler, J., Marrack, P. T cells compete for access to antigen-bearing antigen-presenting cells. J. Exp. Med., 192:1105-1113, 2000.

18. Alexander, J., Oseroff, C., Dahlberg, C., Qin, M., Ishioka, G., Beebe, M., Fikes, J., Newman, M., Chesnut, R.W., Morton, P.A., Fok, K., Appella, E., Sette, A. A decaepitope polypeptide primes for multiple CD8+ IFN-gamma and Th lymphocyte responses: evaluation of multiepitope polypeptides as a mode for vaccine delivery. J. Immunol., 168:6189-6198, 2002.

FIGURE LEGENDS

Table 1. *Immmunodominant HER-2/neu peptide epitopes determined by in vivo immunogenicity also bind class II molecules with high affinity.* Shown is the number and percentage of patients who developed specific T cell responses to 9 HER-2/neu peptides (S.I.>2) when those peptides were administered as a vaccine (1). The number and percentage of HLA-DR alleles associated with *in vitro* high binding affinity of the 9 HER-2/neu peptides are also shown. Data is based on analysis of 14 HLA-DR alleles.

Table 2. *Binding affinities of HER-2/neu peptides with common class II alleles.* The binding specificities of 14 HLA-DR alleles for nine different HER-2/neu derived peptides are shown. Peptides which bind at 1500 nM are considered to bond HLA-DR with high affinity (shaded boxes).

Table 3. *Patients enrolled had MHC Class II alleles capable of binding HER-2/neu immunizing peptides.* Shown are the HLA-DR allele types of patients immunized on this study. Bold checkmarks (✓) represent DR allele types for individual patients. The HER-2/neu peptides associated with high affinity binding are listed above the appropriate alleles.

Figure 1. *The affinity of in vitro peptide binding correlates with in vivo immunogenicity.* Shown is the correlation coefficient for the percentage of high binding DR alleles for a given peptide and the percentage of patients who developed immune responses after active immunization in a previous study (1).

Figure 2. *The minority of patients immunized with the vaccine containing peptides associated with high affinity binding to multiple DR alleles developed HER-2/neu peptide-specific T cell immunity and none developed HER-2/neu protein-specific T cell immunity.* Shown are pre- and post-immunization peptide- and protein-specific T cell responses. Open circles represent pre-immunization T cell response. Solid black circles represent post-immunization T cell response. The dotted line defines mean and 2 standard deviations of 30 aged matched controls.

Figure 3. *The minority of patients immunized with the vaccine containing peptides associated with high affinity binding to multiple DR alleles developed HER-2/neu peptide-specific antibody immunity and none developed HER-2/neu protein-specific antibody immunity.* Shown are pre- and post-immunization peptide- and protein-specific antibody responses. Open circles represent pre-immunization antibody response. Solid black circles represent post-immunization antibody response. The dotted lines define mean and standard deviations of controls for HER-2/neu peptide and protein antibody assays.

HER-2/neu Peptides	Number (%) of patients with SI>2 to HER-2/neu peptides after vaccination	Number (%) of DR alleles associated with HER-2/neu MHC peptide binding
p42-56	7/13 (54)	3/14 (21)
p98-114	10/13 (77)	9/14 (64)
p328-345	5/13 (38)	0/14 (0)
p369-386	12/14 (86)	5/14 (36)
p688-703	5/14 (38)	1/14 (7)
p776-790	11/11 (100)	9/14 (64)
p927-941	8/11 (73)	0/14 (0)
p971-984	9/14 (64)	4/14 (29)
p1166-1180	6/11 (55)	1/14 (7)

Table 1

HER-2/neu Peptides														
	p776 p369 p98	p98	p776 p98		p776	p369 p98	p369	p98	p776	p776 p369 p98	p776 p369 p98	p776 p98	p776 p98	
HLA-DR Alleles														
	DRB1*0101	**DRB5*0101**	**DRB1*1501**	**DRB1*0301**	**DRB1*0404**	**DRB1*0405**	**DRB1*0401**	**DRB3*0101**	**DRB1*1101**	**DRB1*1302**	**DRB1*0701**	**DRB1*0901**	**DRB4*0101**
Subjects													
0275	✓										✓		✓
4998			✓					✓					✓
1518					✓						✓		✓
9975							✓	✓					✓
7695		✓	✓	✓				✓					
4607			✓										✓
0232				✓				✓					✓
3738		✓	✓		✓								✓

Table 3

HLA-DR Alleles

HER-2/neu Peptides	DRB1*0101	DRB5*0101	DRB1*1501	DRB1*0301	DRB1*0404	DRB1*0405	DRB1*0401	DRB3*0101	DRB1*1101	DRB1*1302	DRB1*0701	DRB1*0802	DRB1*0901	DRB4*0101
p42	**304**	7087	**359**	37552	2741	3593	9417	107066	2518	2879	27027	5384	<7700	**163**
p328	13605	17580	18607	<170000	<96000	16530	<19000	111359	<61000	7611	37147	<64000	<7800	29111
p98*	**5**	**46**	**31**	11287	2929	**1024**	8389	**1435**	2104	**5**	**12**	6325	**590**	**120**
p776*	**19**	14197	**46**	167949	**49**	4156	1570	31601	**696**	**955**	**190**	**1317**	**839**	**316**
p1166	10103	11337	23264	134367	103285	<29000	<22000	8733	<53000	**835**	25988	<75000	<7800	25739
p369*	**597**	8008	2264	74162	1897	**37**	**1195**	43745	<53000	**1073**	**377**	<75000	<7800	10020
p971	**201**	3188	7891	**1026**	4882	15120	**120**	14906	4573	<71000	21259	4082	<7800	**970**
p688	11804	<13000	<120000	151105	96140	15520	10659	45269	32468	10594	<68000	<750000	<7800	**1297**
p927*	>20000	>20000	>20000	>20000	>20000	>20000	>20000	>20000	>20000	9409	>20000	>20000	>20000	>20000

* Peptides comprising the vaccine used in the current study

Table 2

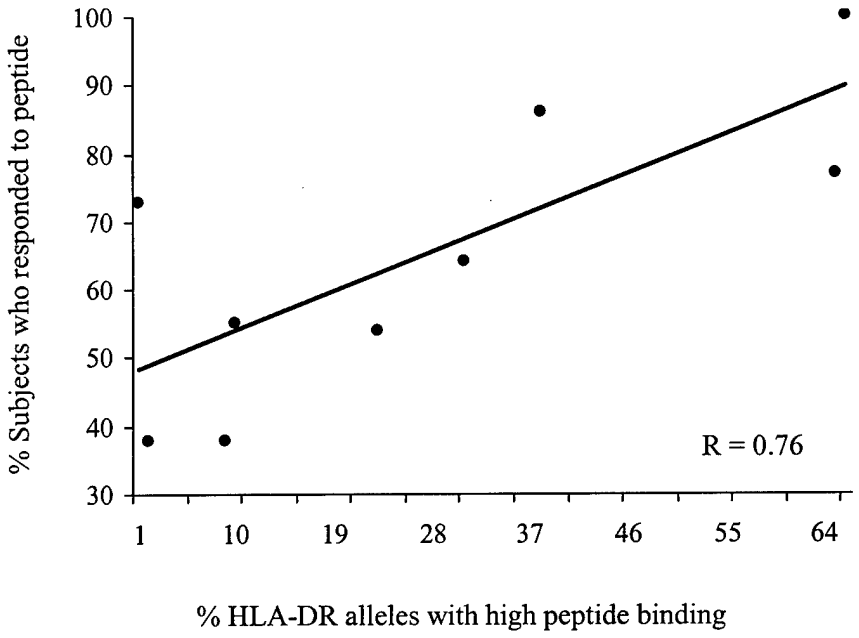
% Subjects who responded to peptide
100
90
80
70
60
50
40
30
1
10
19
28
37
46
55
64
R = 0.76
% HLA-DR alleles with high peptide binding

Figure 1

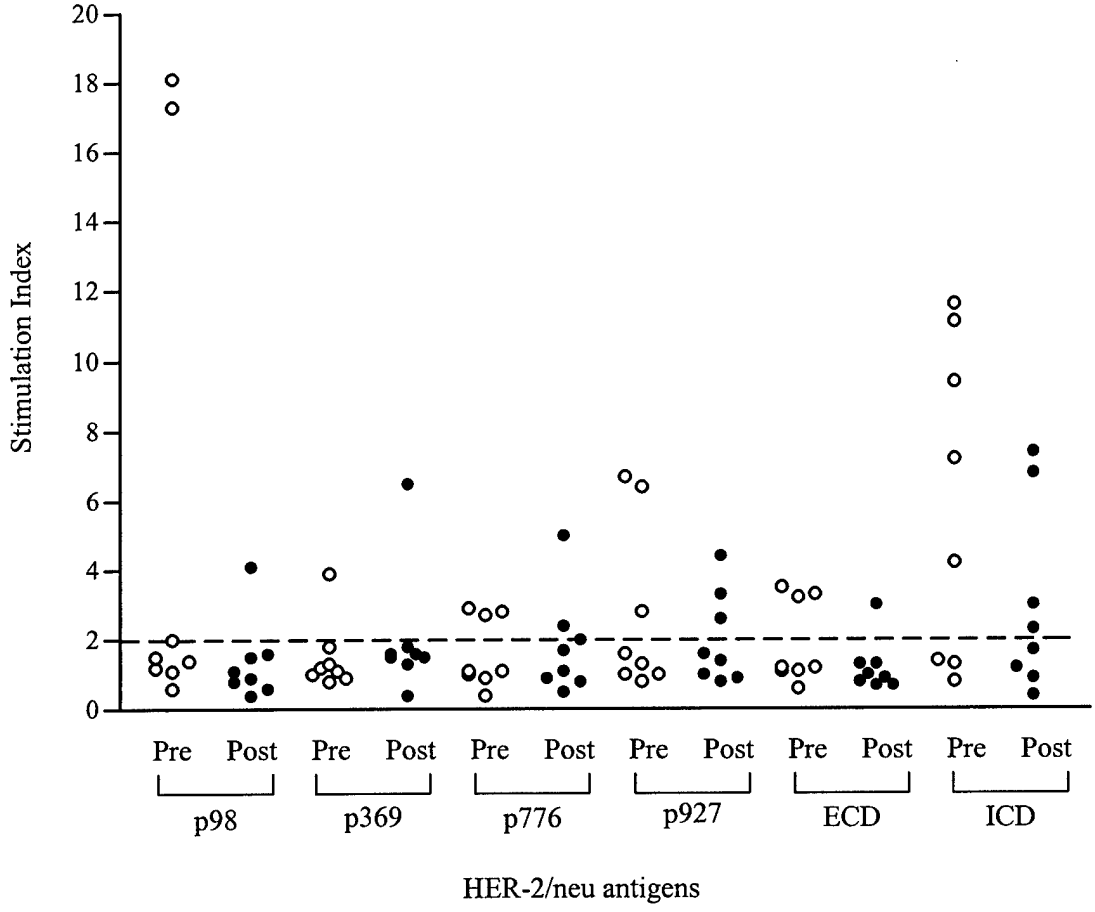
Stimulation Index
20
18
16
14
12
10
8
6
4
2
0
Pre
Post
Pre
Post
Pre
Post
Pre
Post
Pre
Post
Pre
Post
p98
p369
p776
p927
ECD
ICD
HER-2/neu antigens

Figure 2

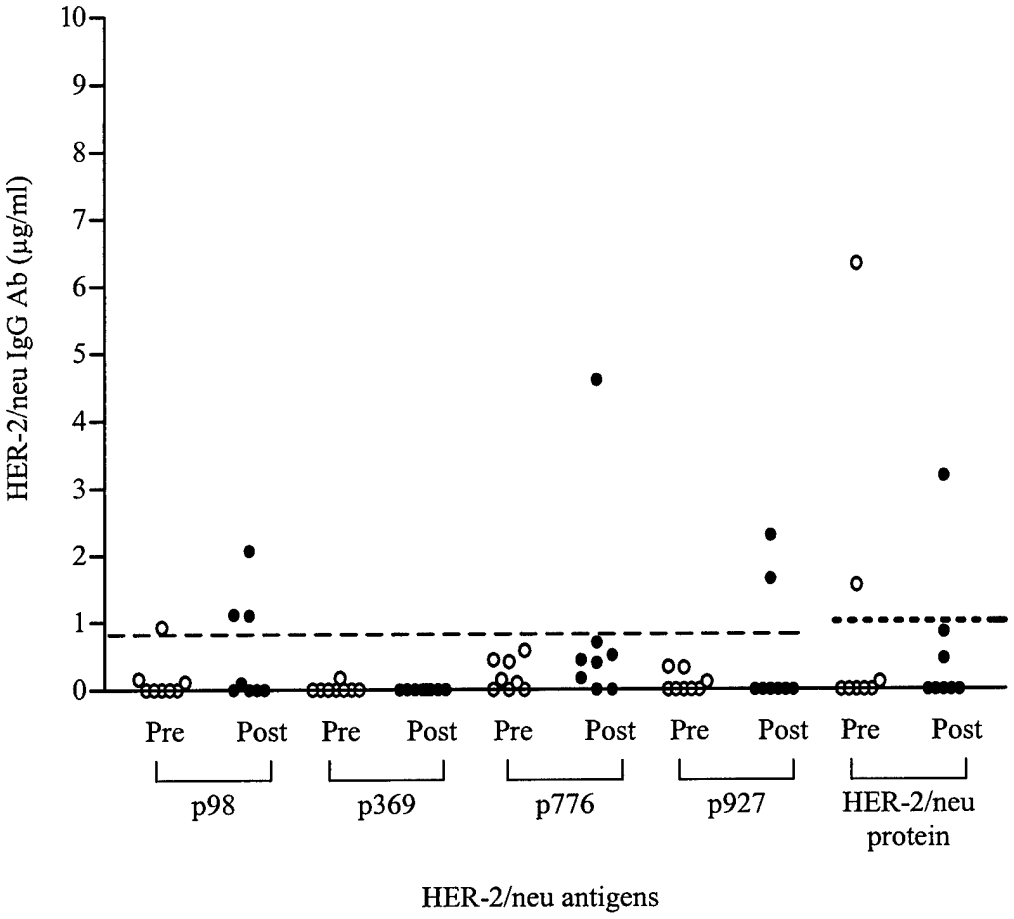
HER-2/neu IgG Ab (µg/ml)
10
9
8
7
6
5
4
3
2
1
0
Pre
Post
Pre
Post
Pre
Post
Pre
Post
Pre
Post
p98
p369
p776
p927
HER-2/neu protein
HER-2/neu antigens

Figure 3

Immunologic Principles and Immunotherapeutic Approaches in Ovarian Cancer

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Abbreviations: APC: Antigen presenting cell

CTL: cytolytic T lymphocyte

DC: Dendritic cell

ip.: intraperitoneal

FBP: folate binding protein

GM-CSF: Granulocyte-macrophage colony-stimulating factor

HLA: Human leukocyte antigen

IFN-α: Interferon alpha

IFN-γ: Interferon_gamma

IL-12: Interleukin 12

IL-2: Interleukin 2

iv.: intravenously

MHC: Major histocompatibility complex

NK: Natural killer cells

NKT: Natural killer T cells

STn: sialyl-Tn

TAL: tumor associated lymphocytes

TIL: tumor infiltrating lymphocytes

Synopsis: Ovarian tumors are immunogenic and several ovarian-related antigens have been identified. In addition, the immunologic characteristics of the tumor microenvironment that may affect ovarian cancer growth are becoming increasingly understood. The type of immune-based approach selected to treat ovarian cancer will depend on the tumor burden. For minimal disease states, active vaccination may be useful for generating adequate protection from relapse. However, for more advanced stage disease states, more rigorous strategies may need to be applied such as adoptive T cell therapy, cytokine infusions, antibody therapy or a combination of different techniques. The immunosuppressive environment observed during advanced malignancy needs to be reversed for improved efficacy of immune-based therapies.

I. Introduction

Immune-based therapies for the treatment of ovarian cancer are now being designed to circumvent specific biologic problems that have been identified from studies of the native immune response to ovarian cancer. Advances in basic immunology over the past decade have resulted in the development of immune-based therapies for ovarian cancer. Perhaps the most important advances are the understanding that ovarian tumors are immunogenic and the identification of tumor-specific antigens. Some of the identified tumor antigens are important for malignant transformation while and others enhance growth, metabolism, invasion or metastasis of tumors. Immune-based therapies targeting these antigens may result in the eradication of tumor cell clones that drive the initiation of malignancy, or help eradicate minimal residual disease after apparently successful conventional treatments.

The roles of the immune and tumor microenvironments in ovarian cancer greatly influence our approach to the design of immunotherapies. In order to augment the immune response to ovarian cancer antigens, not only is the stimulation of both CD8 cytotoxic T cell (CTL) and CD4 helper T cell immunity critically important, tumor-induced immunosuppression must also be overcome. Immune-based therapies are being applied clinically, both for treatment and prevention. Vaccines targeting tumor antigens are increasingly being viewed and studied as chemopreventive agents designed to protect against cancer relapse or development. Established disease is being treated with immune-based approaches, such as monoclonal antibody therapy, adoptive immunotherapy, and intraperitoneal (ip.) cytokine therapy.

II. Ovarian cancer tumor antigens

Malignant transformation results in altered expression of many genes that are related to normal cell growth control and differentiation. A variety of mechanisms are responsible for this altered expression including gene amplification, somatic DNA mutation and gene translocation. Overexpression can result in immune recognition and several tumor antigens have been identified by virtue of specific immunity induced in patients with ovarian tumors. The majority of tumor antigens expressed are non-mutated, therefore, they are self antigens. Examples of some of the well-studied tumor antigens that have been identified and characterized in ovarian cancer are the overexpressed proteins, folate binding protein (FBP), HER-2/neu, MAGE-1, and MUC-1. Targeting immune-based therapies against these tumor antigens is challenging because tolerance to self antigens must be overcome.

In addition to non-mutated antigens, other antigens harbor mutations that can cause abnormal function as well as overexpression. The tumor suppressor gene, p53, is an example of one of these proteins and is often observed in ovarian cancers. Defects in post-translational modifications can also result in the appearance of tumor antigens. For example, glycosylation of proteins is often altered during malignancy. Sialyl-Tn (STn), observed at high frequency on ovarian cancers, is a well-characterized aberrantly expressed carbohydrate antigen that is recognized by the immune system.

One of the more extensively studied ovarian cancer antigens is FBP, a membrane-associated glycoprotein that mediates the intracellular transport of folates. FBP was initially identified as a monoclonal antibody-defined antigen in placenta and trophoblastic cells but rarely found in other issues [1, 2]. FBP is expressed in greater than 90% of ovarian carcinomas [1, 2]. *In vitro* quantitative autoradiographic studies have shown FBP to be overexpressed up to 80-90 fold in non-mucinous ovarian carcinomas compared with normal ovarian tissue [2]. This enhanced expression at the cell surface of ovarian cancers has led to development of FBP as a target for immune-based therapies. FBP is a naturally immunogenic tumor antigen. For example, Peoples and colleagues demonstrated that two FBP peptides (E39 and F41) could be recognized by ovarian tumor-associated lymphocytes (TAL). Stimulation of TAL with these peptide epitopes resulted in antigen-specific proliferation, cytokine (IFN-γ) release and enhanced cytoxicity toward antigen-bearing autologous tumor cells [1]. An equally important property of FBP is that *in vitro* and *in vivo* evidence suggests that it confers some advantage to tumor cell growth. Bottero and colleagues have shown that NIH 3T3 cells transfected with FBP maintained a rapid growth rate *in vitro* longer in physiologic concentrations of folate compared to nontransfected NIH 3T3 cells. In nude mice, FBP-transfected NIH 3T3 cells grew to a 3-fold greater weight over the same time period compared to nontransfected cells [3]. FBP has been and continues to be the target of various immune-based strategies as will be discussed below.

The HER-2/neu protein is a member of the epidermal growth factor receptor family and consists of a cysteine-rich extracellular ligand binding domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity [4, 5]. HER-2/neu is a self protein that is expressed at low levels in a variety of tissues of epithelial origin and plays a fundamental role in cellular proliferation and differentiation in fetal development. In adults, the HER-2/neu gene is present as a single copy in normal cells; however, amplification of the gene and resultant protein overexpression is seen in various cancers including ovarian. The overexpression of HER-2/neu in ovarian cancer was initially observed in 10-30% of newly diagnosed primary ovarian cancers of all stages [6]. More

recently, HER-2/neu overexpression has been found to be more frequent in ovarian cancers relapsing after chemotherapy [7]. Recent studies by Hellstrom and colleagues demonstrated that tumor cell lines established from ovarian tumors as well as tumor cells from malignant ascites obtained at second surgery after relapse, overexpressed HER-2/neu protein as assessed by flow cytometry [7]. This was true despite the primary tumors being HER-2/neu negative upon initial immunohistological analysis suggesting a shift from HER-2/neu negative to positive when the tumor had regrown after chemotherapy [7]. Thus, it is possible that overexpression of HER-2/neu becomes more common as ovarian carcinomas progress, and therefore, anti-HER-2/neu immune-based therapies and vaccines may be beneficial in patients with ovarian cancer including those with negative tumors at primary surgery.

The MAGE gene family comprises a series of 12 closely related genes that are expressed in a variety of tumors [8]. These proteins are part of a larger family of cancer-testis antigens. Some recent investigations suggest that MAGE gene products may be involved in cell cycle regulation, although their exact role in cell biology remains to be defined [9]. These proteins have been studied extensively in the case of melanoma and are known to be immunogenic, making them attractive targets for immunotherapies. MAGE-1 and MAGE-3 encode peptide antigens that are presented in association with HLA class I molecules and are recognized by CTLs and could be targets for specific immunotherapy. Studies have demonstrated MAGE gene expression in ovarian cancer as well as other related tumor antigens, BAGE and GAGE [10]. Gillespie and colleagues found expression of the MAGE-1 gene in 56% of malignant ovarian tissue specimens with preferential expression in serous tumors (71%) and relatively infrequent expression (22%) in other tumors of epithelial origin [8]. In addition, analysis of MAGE-1 expression in serous cystadenocarcinomas revealed a trend toward expression in early stage disease (6/6 stage I lesions and 4/8 stage II, III, and IV lesions) [8]. These findings suggest the potential for expanding MAGE peptide vaccine studies to include some forms of ovarian cancer, specifically serous cystadenocarcinomas.

The MUC-1 gene encodes a high molecular weight cell-surface glycoprotein with a complex cytoplasmic domain thought to be involved in signal transduction [11]. MUC-1 is expressed by most glandular epithelial cells and is often overexpressed by epithelial cancers, specifically breast and ovarian cancer. It has been implicated as an important modulator of adhesion and metastasis [11]. Both CTL and CD4 T helper cells specific for the MUC-1 core peptide have been observed in multiple studies of immunity to carcinomas of the ovaries, breast and other tissues [12-14]. This has generated interest in evaluating the utility of targeting MUC-1 in specific

immunotherapies. Although the majority of MUC-1-specific immunotherapies are directed toward colorectal and breast cancers, MUC-1 also appears to be a promising target in ovarian cancer.

Malignant tissues may express mutated or wild type p53, either of which can be immunogenic and thus a target for immune-based therapies. Mutations of the p53 tumor suppressor gene lead to a functionally inactive mutant p53 protein and are common, in ovarian cancers, occurring in approximately 50% [15]. Ovarian cancers are known to be among the most immunogenic malignancies inducing an anti-p53 autoantibody response [16]. The prevalence of anti-p53 antibodies in patients with invasive cancer was 19%, whereas no circulating anti-p53 antibodies were detected in patients with borderline or benign lesions. Anti-p53 antibodies were detectable only in patients with p53 protein overexpression in their tumors, and the presence of anti-p53 antibodies correlated with tumor stage and grade and shortened overall survival and relapse-free survival [17]. Naturally expressed peptide epitopes have been identified for the development of active immunization strategies aimed at augmenting p53-specific T cell immunity [18, 19].

Carbohydrate epitopes can be immunogenic in malignancies due their overexpression or their cellular distribution. STn, a disaccharide, is a blood group-related carbohydrate antigen that belongs to the mucin core family. It is has been reported to be overexpressed in 31 to 100% of ovarian carcinomas and has been shown to be an independent predictor of poor prognosis in patients with epithelial ovarian cancer. Kobayashi and colleagues found that levels of STn were significantly higher in the sera of patients with ovarian cancer when compared with levels in benign and healthy controls [20]. In addition, the survival rate for patients with STn negative versus STn positive ovarian tumors was 76% versus 10.8%, respectively and the progression-free interval at 5 years was 51.9% versus 5.4% respectively [20]. Further multivariate regression analysis demonstrated that stage, residual tumor size, positive STn, performance status, and histologic grade were the five important variables for predicting overall survival [20]. Although STn has been an appealing tumor antigen since it was first identified, one of the major issues has been in defining its distribution on ovarian carcinomas in efforts to aid in the choice of antigenic targets for both passive and active immunotherapy. Recent studies examining the distribution of STn and other carbohydrate antigens have demonstrated a significant difference in antigen expression between mucinous and other histological types of ovarian carcinoma [21]. Federici and colleagues found the majority of mucinous tumors to have a strong and relative uniform expression of STn whereas in serous tumors, STn was less frequently expressed or the expression was very heterogeneous [21]. So while STn would be a good target antigen for patients with

mucinous tumors, serous tumors may require distinct immune-based approaches. In serous tumors, other carbohydrate antigens such as Le^y and H type 2, which are highly overexpressed, may be the preferred targets [21].

Several tumor antigens have now been identified that could be targets for immune-based approaches for the treatment and prevention of ovarian cancer. In addition to those antigens that are mutated or result from aberrant posttranslational changes, others are normal but over-expressed and play critical roles in malignant transformation or other important tumor cell processes. Augmenting the immune response to these normal self proteins will require additional efforts to overcome tolerance. As the tumor burden increases, the tumor microenvironment can suppress the immune response. Thus, designing therapies targeting specific antigens in patients with significant disease burden will involve overcoming immunosuppression.

III. The immunosuppressive ovarian cancer microenvironment

The tumor microenvironment is hostile to immune effectors, and tends to induce immune tolerance, suppression or anergy. Immune evasion may be due to reduced processing or presentation of tumor-associated antigens, inhibition of function of immune effector cells, killing of immune effector cells, or reprogramming of immune effector cells to mediate inappropriate immunity. To effect these perturbations in immunity, ovarian carcinomas secrete a multitude of immunosuppressive factors, including into the serum [22]. These immunosuppressive factors can directly inhibit an anti-tumor response or promote the influx of immunoregulatory cells. Ovarian tumors also produce a significant array of cell-bound ligands that perturb immune functioning following direct contact with immune effectors.

The ovarian immune microenvironment involves a complex network of cytokines and soluble effectors that suppress immunity. Recent work, for example, demonstrates that ovarian tumors express significant amounts of the chemokine, stromal-derived factor-1 (SDF-1) [23]. Ordinarily, SDF-1 is involved in normal embryogenesis, cardiogenesis, and hematopoiesis [24]. However, its role becomes pathogenic when overexpressed by tumor cells because it attracts immature, immune inhibitory dendritic cells (DC). Specifically, SDF-1 attracts precursor plasmacytoid dendritic cells (pPDCs) through pPDC expression of the chemokine receptor, CXCR4 [23]. SDF-1 increases pPDC expression of adhesion molecules including the integrin CD49d. Expression of these cell surface molecules facilitates adhesion/transmigration of pPDC into the tumor microenvironment [23]. pPDCs are able to suppress the development of T cell-mediated immunity. Recruited pPDCs induce the secretion of the

immunosuppressive cytokine, IL-10, from T cells into the tumor microenvironment. IL-10 is an anti-inflammatory cytokine and inhibits tumor-specific T cell proliferation, as well as infiltration and activation of antigen-presenting cell (APC) that are critical in the initiation of an immune response. Ordinarily IL-10 would induce apoptosis of the pPDC but the tumor-derived SDF-1 strongly inhibits IL-10 induced apoptosis thereby maintaining a persistent pool of intraperitoneal pPDC. Thus, SDF-1 elaborated by the tumor helps circumvent activation of potentially therapeutic immunity by causing the influx and maintenance of immunosuppressive pPDCs. Boosting ovarian-specific immunity will require that this suppressive mechanism is blocked. Interruption of IL-10 production or signals, or of SDF-1/CXCR4 interactions or their downstream signals are potential strategies to attempt to block immunosuppression. SDF-1 and IL-10 represent only 2 of many immunosuppressive soluble factors specific for ovarian cancer. Other soluble factors produced in the ovarian tumor microenvironment include TGF-β and IAP, both of which also impair tumor-antigen-specific T cell immunity [25]. VEGF is also secreted by ovarian tumors and in addition to its angiogenic and permeability effects, it is known to directly inhibit the maturation of DC resulting in inhibition of expansion of anti-tumor immune effectors [26]. Thus, many soluble factors are secreted by the ovarian tumor that can dampen the immune response.

In addition to immature DC, another immunosuppressive cell that may play a role in preventing ovarian tumor from being recognized by the immune response is the $CD4^+CD25^+$ T regulatory (Treg) cell. Tregs are potent suppressors of CD4 and CD8 T cells both *in vivo* and *in vitro*. Tregs are thought to play a critical role in maintaining tolerance to self antigens. Their absence is associated with the development of autoimmune and inflammatory diseases in animal models [27]. A significant fraction of CD4 T cells in ascites of patients with ovarian carcinoma express the $CD4^+CD25^+DR^+$ Treg phenotype and could contribute to pathogenesis [28]. Tregs may inhibit attempts to induce active immunity or could prevent the adequate function of adoptively transferred T cells. Strategies could be designed to eliminate peritoneal associated Tregs prior to active immunization, such as with the use of anti-CD25 antibodies.

Ovarian tumors express cytokine receptors and cytokines released into the tumor microenvironment may augment the growth of ovarian tumors. For example, IL-6 has been implicated in the pathogenesis of many cancers including ovarian. IL-6 is secreted by macrophages, vascular endothelium, and activated T cells in the immune microenvironment. The major roles of IL-6 are to induce production of acute phase plasma proteins (e.g. fibrinogen) and to promote the differentiation of B cells. High levels of IL-6 have been observed in peritoneum and

serum of patients with ovarian cancer. In the serum, the levels of IL-6 are at 100-fold higher levels as compared to healthy disease-free individuals [29]. Elevated IL-6 concentrations in the blood correlate with poor disease-free and overall survival in ovarian cancer patients [29]. IL-6 directly enhances invasiveness of ovarian tumor cells by improving the tumor cell's ability to attach to and migrate into other tissues [30]. Conversely, elevated IL-6 concentrations in the peritoneum are correlated with increased numbers of antigen-specific B and memory T cells [31]. The dichotomy of effects of IL-6 in the ovarian cancer microenvironment underscores the complex interactions present in the peritoneum. While increased levels of a cytokine such as IL-6 may potentially benefit the development of an immune response, ovarian tumors themselves can respond directly to IL-6 for enhanced immune evasion and pathogenesis.

Ovarian tumors also express cell surface receptors that can also impact the generation of an immune response. For example, ovarian tumors express several apoptosis related proteins, notably Fas, FasL and B7-H1. The Fas/FasL system plays an important role in limiting the immune response. Fas/FasL interactions result in apoptosis of Fas-expressing cells mediated by cross linking of Fas by FasL. Baldwin and colleagues observed that despite abundant expression of Fas, primary ovarian tumor cell lines are resistant to FasL-mediated apoptosis [32]. This resistance likely plays a significant role in immune evasion, since the Fas/FasL is a major mechanism by which CTL are able to directly kill tumor cells. Ovarian tumors also express FasL which promotes T cell dysfunction and apoptosis. Rabinowich and colleagues reported that ovarian tumors express FasL *in situ* and *in vitro*, and are capable of inducing death of Fas-expressing T cells [33]. They also observed that engagement of Fas on T cells by FasL-expressing ovarian tumor cells played a role in down-regulation of T cell cytoplasmic CD3-zeta and cell surface CD3-epsilon chains, both of which are required for T cell activation. B7-H1 is a member of the B7 family of co-stimulatory molecules and is overexpressed on ovarian tumors [34]. Recent studies have shown that tumor cell-associated B7-H1 increases the apoptosis of antigen-specific T cells. Thus, ovarian tumors are well-equipped with redundant mechanisms with which to destroy tumor-specific T cells by inducing apoptosis.

Overall, the tumor microenvironment defies the generation of an effective anti-tumor immune response using variety of different cellular and molecular mechanisms. At present it is unknown what contribution each individual suppressive mechanism makes to the overall hostile environment. It is clear that prior to immunologic intervention (e.g. active immunization) at least some of these suppressive mechanisms will need to be overcome or

reversed. Our improved understanding of the tumor microenvironment will allow us to develop the appropriate procedures and tools to accomplish this objective.

IV. Effectors targeted in immune-based approaches

An effective anti-tumor immune response will consist of complex interactions among multiple cellular and soluble immune effectors. Effector cells that may induce or mediate an anti-tumor immune response include CD4 T cells, CTL, NK cells, NKT cells, and DC. Several studies have demonstrated that ovarian cancer-specific immune effectors are elicited *de novo* in response to the disease, prior to immunologic intervention.

CTL are a subset of T lymphocytes that possess the ability to home to and kill tumor cells by specifically recognizing 8-10 amino acid fragments of antigenic proteins presented by MHC molecules. CTL have been thought to be primary mediators of antitumor immunity. In studies in ovarian cancer patients, CTL established *in vitro* demonstrate significant levels of cytolytic activity against known ovarian cancer antigens such as FBP and HER-2/neu [1, 35] . Therefore, CTL specific for ovarian cancer antigens do exist naturally *in vivo* in patients with ovarian cancer and may have a biologic effect on the tumor, but clearly are not sufficient in eradicating cancer. This lack of sufficient immunity has been documented in patients with cancer. For example, in breast cancer patients, pre-existing HER-2/neu-specific immunity is low, at fewer than 1:100,000 of circulating T cells [36]. While it was initially thought that augmenting CTL alone might lead to tumor eradication, recent developments suggest that CTL alone cannot constitute a complete anti-tumor response and that additional immune effectors such as CD4 T cells are needed for completing the appropriate immune milieu.

The CD4 or T helper cell represents another major subset of the immune system's T lymphocyte population. The CD4 T cell is critical in controlling the activation and persistence of the immune response against viral infections [16]. The CD4 T cell elaborates a variety of cytokines and chemokines that cause influx of many other immune effectors such as macrophages, neutrophils, B cells, and CTL. Although no perfect animal model exists for ovarian cancer, the interplay between CTL and CD4 T cells in eradicating cancer has recently been evaluated in neu transgenic (neuTg) mice. These mice express transgenic rat neu under control of the MMTV promoter and develop neu-overexpressing epithelial breast cancer similar to those that occur in humans [37]. Also, like in humans, transgene expression of neu confers immunologic tolerance to neu. While tolerant, neuTg mice can be vaccinated against neu and develop both neu-specific T cells and neu-specific IgG [38]. The resulting vaccine-

induced neu-specific immune response, delays the onset of the spontaneous neu-mediated tumors. Depletion studies demonstrated that not only are CTL required for therapeutic efficacy, but also CD4 T cells. The importance of CD4 T cells in the evolution of the tumor-specific immune response is underscored by recent studies demonstrating that CD4 T cells can initiate a *de novo* CTL response. As an example, infusion of a tumor antigen-specific Th1 CD4 T cell clone resulted in the development of a CTL anti-tumor immune response, presumably due to CD4 T cells secreting cytokines appropriate to enhance the function of local APC to cross-present tumor antigen to endogenous CTL [39]. CD4 T cells can also produce cytokines that attract macrophages, DC, and eosinophils to promote an inflammatory environment [40]. The role of CD4 T cell help in the immune response has led to the identification of MHC class II binding epitopes of well-known human ovarian tumor antigens such as HER-2/neu [41].

The tumor-specific immune response can not be elicited without effective APC processing and presentation of tumor proteins to T cells. DC are potent APC and are important in initiating a T cell response. DC exist in peripheral tissue in an immature state awaiting encounter with antigen. Immature DC are efficient at antigen uptake and processing but are poor at activating T cells because MHC molecules are in intracellular depots rather than at the cell surface, and T cell costimulatory molecule expression is low [42]. Following exposure to antigen or proinflammatory stimuli, DC migrate to regional draining lymph nodes (DLN) and it is during migration that DC attain the ability to present antigen, i.e. undergo maturation. Antigen-specific T lymphocytes are then stimulated by direct contact with the DC. DC can acquire antigen by various means including, endocytosis of soluble protein, phagocytosis of tumor-derived exosomes and receptor-mediated uptake of apoptotic bodies. DC function is heavily influenced by the tumor microenvironment wherein cytokines can act at many different levels including APC differentiation [43], lifespan [44], migration [45], and antigen-presentation [46]. Several cytokines are known to promote dendropoiesis and include GM-CSF and Flt-3 ligand, both of which have been shown to mediate tumor regression *in vivo* [47, 48]. While the administration of either GM-CSF or Flt-3 ligand increases the total number of circulating DC, GM-CSF promotes myeloid dendropoiesis exclusively while Flt3 ligand promotes both myeloid and lymphoid dendropoiesis [43]. Aside from the immature precursor plasmacytoid DC that inhibit immune responses in ovarian cancer, mature DC with T cell activating properties are absent in the peritoneal cavity ovarian cancer patients [23]. The reason for their absence is unclear but may be related to active immunosuppression. Thus efforts have been or are being applied in ovarian cancer patients, as will be discussed below, to mobilize DC either *in vivo* or *in vitro* with the use of cytokines that promote their differentiation and present tumor antigens to T cells.

Immune effectors other than T cells or DC may also be important in the immune response against ovarian cancer. The natural killer (NK) cell recognizes tumors in a non-MHC-restricted fashion. NK cells are large granular lymphocytes that lack surface IgG and T cell markers. NK cell cytolytic activity is inhibited through interaction of the NK expressed KIR receptor with MHC class I molecules. The role of NK cells in the immune response, therefore may be to eliminate dysfunctional cells that lose MHC class I expression. Immunohistochemical analysis of human ovarian tumors shows that while most of the tumor cells express MHC class I, a small fraction are negative and could be targets for NK cells [49]. NK cells also play a significant role in the adaptive immune response by lysis of antibody-coated pathogens or tumor cells [50]. Upon activation, NK cells secrete IFN-γ and TNF-α and promote a Th1-type T cell response [50]. In a SCID mouse model of human ovarian cancer, IL-12 injections significantly inhibit ovarian tumor growth which is associated with an increased infiltration of NK cells [51]. These results suggest that NK cells may be a component of a therapeutic anti-tumor immune response.

A newly identified cell of a lymphoid lineage, the Vα14 NKT cell, may also be an effector cell that is important in the immune response against ovarian cancer. The NKT cell expresses typical NK cell surface receptors and a semi-invariant T cell receptor (TCR) encoded by Vα14 and Jα281 [52]. The NKT cell is activated through recognition of glycolipid antigens in association with CD1b, a MHC-like molecule present on APC. NKT cells secrete predominantly IL-4 and IFN-γ and may be important in regulating the Th1/Th2 cytokine phenotype during an immune response against ovarian cancer. Presentation of glycolipids by $CD1d^+$ DC to NKT cells can result in the generation of lymphoma-specific CTL suggesting an important interface between the innate and adaptive anti-tumor immune response [53]. Baxevanis and colleagues have observed that NKT cells can be elicited from ovarian cancer patients using acid extracts from autologous tumor cells [54]. The NKT cell had dual specificity recognizing tumor cells in both an MHC-dependent and MHC-independent fashion. The role of these ovarian-specific NKT cell however remains to be elucidated. Ovarian cancer is known to have altered ceramide and ganglioside synthesis [55]. Presentation of altered glycolipids expressed through CD1b on DC may further enhance the immune response by activating NKT cells. Future immunotherapeutic strategies could also be designed to specifically target this novel immune effector subset.

V. Immune-based strategies for minimal disease.

Despite the fact that many patients with advanced ovarian cancer can be treated into remission, most (~85%) will relapse and eventually die from the disease due to incomplete eradication of microscopic disease [56]. Remission is an ideal time to intervene with active immune-based strategies because the tumor burden and growth are reduced, and therefore the influences of the tumor microenvironment on the immune response are minimized. Active immunization of ovarian cancer patients with vaccines to prevent recurrence is conceptually similar to the use of infectious disease vaccines that are given either as a prophylactic prior to or shortly after exposure to infectious agent. Likewise, cancer vaccines should be used in the prophylactic setting because the magnitude of the immune response induced by vaccine is limited and reaches a plateau, often not of a sufficient magnitude to mediate regression [57].

The goal of vaccination against ovarian cancer is to generate significant immunologic memory capable of eliciting an immune response during antigen exposure that may occur early during relapse. Several different vaccine strategies have been or are currently being tested for efficacy at inducing immune responses against cancer antigens, including dendritic cell (DC)-, peptide-, protein-, whole tumor cell-, viral carrier-, or DNA-based vehicles. Unlike infectious disease vaccines, a major obstacle of cancer vaccines is tolerance [58]. While it is known that patients can have a pre-existent immune response to tumor, tolerance keeps the immune response in check, and most antigens expressed on most cancers are self antigens [36]. Thus, efforts have been applied to identifying how to overcome tolerance with the use of strong adjuvants [59]. Most likely, the best adjuvants are going to be those that directly activate and mobilize DC, the most potent APC of the immune system. DC can directly activate both memory CD4 T helper cells and CTL, both of which are important for the generation of a potentially prophylactic immune response [42]. Activation of DC for presentation of tumor antigen can be accomplished in two different ways. One strategy that has been widely tested is to generate DC *ex vivo*, followed by antigen loading and reinjection. DC can also be directly activated *in vivo* by the application of a variety of exogenous adjuvants, such as GM-CSF [59].

Ex vivo generation of antigen-pulsed DC has been used extensively in vaccination strategies and can result in the generation of substantial long-lived immunity [60]. Relatively few studies, however, have assessed the efficacy of this approach in ovarian cancer. In one report, Brossart and colleagues conducted a small pilot trial to assess both the clinical and immunologic consequences of vaccinating ovarian and breast cancer patients, all with

bulky disease, with one of two vaccines consisting of *ex vivo*-derived DC pulsed with either the HER-2/neu or MUC-1 peptides [61]. Half of the 10 patients vaccinated developed peptide-specific responses as assessed by staining for intracellular IFN-γ production by T cells. The peptide-specific T cells were able to lyse HLA-matched HER-2/neu-overexpressing tumor cell lines, suggesting effective immunization against naturally processed antigen. Vaccination resulted in partial regression in 1 patient, and disease stabilization in another 2 patients. In another study, Hernando and colleagues immunized ovarian cancer patients with autologous DC pulsed with tumor lysates and keyhole limpet hemocyanin (KLH) as a helper antigen [62]. The majority (6/8) demonstrated immunity against KLH but only 2/6 patients generated immunity to tumor lysates. The two patients that generated immunity specifically against tumor had disease stabilization for several months following vaccination. The inability to achieve a vaccinated response in a majority of individuals may be due to tumor-induced immunosuppression. The disappointing clinical results are consistent with results of other dendritic cell-based clinical trials and support the hypothesis that vaccines are prophylactic rather than therapeutic agents. Another technique that is being investigated is the direct *in vivo* activation and mobilization of DC with adjuvants, such as GM-CSF. When delivered intradermally, GM-CSF can act as a recruitment and differentiation factor for the skin-based Langerhans' cell [63].

Our laboratory has conducted phase I clinical vaccine trials using peptide-based vaccines in ovarian cancer patients with HER-2/neu-overexpressing tumors who had been maximally treated [64]. The patients, in general, had only minimal or no evidence of disease and had been off of chemotherapy for at least 30 days. The HER-2/neu peptide-based vaccines consisted of HLA-class II binding peptides admixed with GM-CSF. Our immunization strategies were designed to elicit a HER-2/neu-specific CD4 T helper response. Our view is that generation of a robust CD4 T cell response can lead to the generation of an endogenous memory response that could include the generation of memory B cells and CTL. Patients were immunized once a month for 6 months. Five of 11 (46%) patients with ovarian cancer completed the planned 6 vaccinations. The majority of patients were immunized to at least one of the peptides in their vaccine. Overall, 7 of 11 (64%) patients developed peptide-specific immune responses. In addition, all 5 patients who completed the full course of vaccinations developed HER-2/neu protein-specific immunity after peptide immunization. These results with peptide vaccines show that T cell immunity can be generated in ovarian cancer patients and vaccines can be developed that are capable of stimulating natural

processing and presentation of antigen *in vivo*. Furthermore, these results set the stage for future clinical trials evaluating whether immunization can confer protection from relapse.

Currently, a number of other vaccination strategies are being tested in patients with minimal or no ovarian disease burden. Holmberg and colleagues investigated whether the STn vaccine Theratope could protect breast and ovarian cancer patients from relapse following high-dose chemotherapy and autologous stem cell rescue [65]. Patients were immunized with 5 vaccinations of Theratope at a median of 127.5 days following stem cell transplant. Overall survival analysis indicated some improvement in survival among those patients that had chemotherapy-responsive disease (i.e. low-to-intermediate risk for relapse). The cohorts were too small to reach statistical significance, when compared with control populations. However, relapse hazard was significantly lower in patients with higher levels of tumor-specific killing as assessed by *in vitro* cytolytic assays.

Active immunization with the use of vaccines targeting single antigens has shown that cancer patients can be immunized against tumor antigens. Targeting single epitopes or antigens has led to the generation of antigen-loss variants [66, 67]. From an immunologic standpoint, combination vaccines that target multiple immunogenic proteins may demonstrate improved clinical outcomes due to increased numbers of tumor-specific immune effectors. A number of potential options exist for the generation of immunity that targets multiple antigens. The generation of an immune response to multiple antigens could also be achieved naturally by epitope spreading, which may occur by vaccination in the presence of some tumor. Epitope spreading is an amplification of the immune response that results from extending immunity from one antigen to other secondary antigens also expressed by the tumor cells. Epitope or determinant spreading, is a phenomenon first described in autoimmune diseases including Theiler's murine encephalomyelitis virus-induced demyelinating disease, murine experimental autoimmune encephalitis, and diabetes in non-obese diabetic mice [68]. As a result of an inflammatory response and resulting tissue damage, debris is taken up and other antigens are cross-presented by APC to CD4 T cells in the regional lymph nodes. These newly recruited antigen-specific CD4 T cells could further exacerbate tissue destruction. In the cancer vaccine setting epitope spreading has been observed. In the DC-based vaccine study by Brossart and colleagues described above, in some of the responding patients intermolecular epitope spreading occurred to tumor antigens CEA and MAGE-3, indicating potential inflammation at the tumor site as a result of vaccination [61]. Our group has also observed epitope spreading in our HER-2/neu peptide-based vaccine trials [64]. Eighty-four percent of patients who completed the course of vaccinations developed intramolecular epitope spreading to HER-2/neu

peptides not contained within their vaccine preparation. The epitope spreading was significantly associated with the development of HER-2/neu protein-specific immunity, consistent with the notion that epitope spreading is a function of natural endogenous processing of antigen.

Multi-antigen vaccines can be constructed combining multiple peptides or proteins. Alternatively, DNA or mRNA immunization represents an appealing approach because multiple antigens could be encoded into a single construct. DNA or mRNA would obviate the need for identification of MHC class I or II binding epitopes and would likely be easier to construct than protein vaccines which, require difficult purification techniques. Whole-tumor cell vaccines have also been used and have shown some clinical efficacy and could lead to the generation of a broader immune response [69]. However, whole tumor cell vaccines require that tumor samples be obtained, an option that is often not feasible such as when patients are referred for vaccination after standard therapy. Ideally, a vaccine to prevent disease recurrence would be easily constructed, highly immunogenic, and accessible to all patients.

V. Immune-based strategies for advanced ovarian malignancy.

In contrast to microscopic or undetectable disease, the treatment of established bulky malignancy will likely be different than strategies required for the prevention of relapse for a number of reasons. First, bulky established malignancy is physically more difficult for immune effectors to reach and eradicate [70]. Second, a large tumor burden is generally more immunosuppressive than smaller burdens [71]. Third, tumor growth may outpace an endogenously generated immune response (e.g. vaccine response). Lastly, large tumors are vastly heterogeneous therefore presenting a greater chance of generating antigen-loss or immune-escape variants [72]. A number of different approaches for the treatment of large tumor burdens are being examined in ovarian cancer and include monoclonal antibody therapy (unconjugated and conjugated), adoptive T cell therapy, and cytokine therapy.

Monoclonal antibody therapy is becoming more widely used in larger tumor burden settings and at present several unconjugated antibodies have been approved for cancer therapy [73, 74]. The therapeutic efficacy of unconjugated antibodies relies on both the antigen-binding domain and the Fc region [75]. The antigen-binding domain can mediate a number of effects including blocking ion channels, altering cell signaling, or promoting internalization of the antigen, all of which may have detrimental effects on tumor cell growth [76, 77]. The Fc receptor is important for mediating antibody-dependent cell-mediated cytotoxicity or complement-dependent

cytotoxicity [78]. When conjugated, monoclonal antibodies can be effective as tissue-specific delivery vehicles for toxins and radiotopes [75].

Compelling evidence suggests that select ovarian cancer patients may benefit from anti-HER-2/neu monoclonal antibody treatment (i.e., trastuzumab). First, like breast carcinoma, HER-2/neu-overexpressing ovarian tumor cells are suppressed *in vitro* by exposure to HER-2/neu-specific antibodies, including trastuzumab [79]. Secondly, a phase I trial specifically inhibiting HER-2/neu function in ovarian tumors using intrabodies have suggested some therapeutic efficacy [80]. In the phase I intrabody trial, study patients with HER-2/neu-overexpressing ovarian cancer were treated with an adenovirus construct encoding for a single chain antibody that binds to the intracellular domain of HER-2/neu. Although no complete regressions were observed, 5 of 13 patients had stabilized disease, indicating that HER-2/neu may play some role in driving tumor proliferation. The use of therapeutic HER-2/neu-specific antibodies may benefit a greater percentage of ovarian cancer patients compared to breast cancer as recent evidence now suggests that a large percentage of advanced stage ovarian malignancies are HER-2/neu-overexpressing.

Other experimental monoclonal antibodies are also being examined for therapeutic activity. Bevacizumab, an anti-VEGF monoclonal antibody, is being tested for efficacy against a variety of cancers including ovarian [81]. VEGF is a proangiogenic growth factor secreted by a number of different tumors and is important in increasing the tumor microvasculature [82]. Preclinical studies have demonstrated that bevacizumab is a potent growth inhibitor in a variety of human cancer xenografts [83]. Tumor growth inhibition is accompanied by reduced vascular permeability, tumor vessel density, and angiogenesis. Early clinical trials evaluating safety have demonstrated some tendency toward an improved response when combined with chemotherapy [81, 84]. In one trial, patients with advanced non-squamous lung cancer were treated with either chemotherapy plus bevacizumab (2 dose arms, 7.5 mg/kg or 15 mg/kg) or chemotherapy alone [84]. The overall response rates for the 7.5 mg/kg arm was 31.8% and for the 15 mg/kg arm, 32.3%, while the response rate of chemotherapy-only arm was 12%. In addition to improving the clinical response rate, the time-to-progression improved in responders from 17 weeks in the chemotherapy alone arm to 32 weeks in the 15 mg/kg arm. However, bevacizumab is associated with serious bleeding toxicity and may not be suitable for all patients such as those with cardiovascular problems [81].

The folate binding protein (FBP), also known as membrane folate receptor, has also been studied as a target for immune-based therapy in ovarian cancer [85]. In the late 1980's several high affinity anti-FBP antibodies were

generated that have been adapted to clinical use [86]. The MOv18 antibody has been studied extensively in patients in both unconjugated and conjugated forms demonstrating some therapeutic efficacy [87-89]. In one study, late stage ovarian cancer patients with detectable disease were treated with a single dose of ^{131}I-conjugated MOv18 30-40 following second-look evaluation [87]. Ninety days after therapy, patients underwent a third-look evaluation. Five patients demonstrated complete responses, 6 patients had stable disease, and 5 others had progressive disease. The responses were durable for at least 10 months and the toxicity of this approach was minimal. In a recent pilot trial, 5 women with residual or recurrent ovarian tumors were given 4 weekly infusions of unconjugated MOv18 [88]. Safety, toxicity, and clinical responses were examined. Although antibody therapy was well-tolerated, no complete responses were observed. Three patients experienced disease stabilization with a mean time to progression of 9 months. The results with MOv18 therapy are encouraging and suggest that antibody therapy targeting FBP may be useful under certain conditions such as when tumor burden is minimal or if the antibody is conjugated to toxins or isotopes. The use of MOv18 in combination with other immune-based strategies may improve efficacy since the data clearly suggest that the antibody is capable of slowing or halting disease during therapy in most patients.

Another approach that is now being widely tested for the treatment of different types of cancer is adoptive T cell therapy [90]. The primary purpose of adoptive T cell therapy is to augment T cell responses to levels greater than that achievable by vaccination alone. Vaccination can increase the number of immune T cells capable of recognizing and responding to antigen. Repeated vaccination further increases the number of immune effector cells, but eventually a plateau of responsiveness is reached, and repeated immunizations do not appreciably change this value [57]. Adoptive immunotherapy may allow levels of immunity to be achieved which could mediate an anti-tumor response. Adoptive transfer of T cells has resulted in the infused cells representing as many as 90% of the host's lymphocytes [91]. Experiments in a murine model of HER-2/neu-overexpressing cancer have shown that vaccination alone, in the neu-transgenic mouse, is effective only for prevention of disease and not for the treatment of established malignancy [92]. Mice immunized with a neu-specific peptide-based vaccine are able to resist a tumor challenge following the course of immunization. In contrast, if vaccination is started on the same day as a tumor implant, tumors grow at the same rate in vaccinated as in control mice. Therefore, *in vivo* expansion of antigen-specific T cells must be limited or even suppressed. One potential method of increasing the number of antigen-specific T cells to the level needed to eliminate tumor is to expand T cells *ex vivo* followed by reinfusion. T

cells derived from neu-transgenic mice immunized against neu were expanded *in vitro* with neu peptides [92]. The neu-specific lymphocytes were then infused into tumor bearing mice resulting in tumor regression. Splenocytes from non-immunized animals were infused as a control and had no anti-tumor effect. Although adoptive immunotherapy has been successful in eradicating established disease in animal models, the approach has not been as successful in human clinical trials [92]. Obstacles to the development of successful T cell therapy for human ovarian cancer have been the (1) lack of defined tumor antigens for expansion of antigen-specific T cells, (2) lack of a detailed understanding of the *in vitro* expansion requirements of T cells that would allow the generation of maximal numbers while retaining optimal antigen-specific function, and (3) lack of understanding the *in vivo* environment necessary for sustaining expansion *in vivo*. Over the past several years however, a number of antigens have been identified, including HER-2/neu, as described above that can improve the *ex vivo* expansion of tumor-reactive T cells. In addition, the cytokine and antigen environments needed to expand a functional anti-tumor population and sustain it *in vivo* have been characterized to some degree [92].

Recent studies from our group have shown that it is technically feasible to readily expand ovarian antigen-specific T cells from patients who have been immunized with peptide-based cancer vaccines [93]. The ease of *ex vivo* isolation and expansion is related to a starting precursor frequency that has been elevated as a result of vaccination [92]. As an example, an ovarian cancer patient developed a significant increase in the CD8 T cell precursor frequency to HER-2/neu HLA-A2 binding peptide, p369-377, after active immunization. T cell clones specific for p369-377 were isolated from the peripheral blood by limiting dilution and characterized. A total of 21 p369-377 clones were generated from this patient and with the exception of two clones, all clones were $CD3^+$. Eleven of the clones were $CD8^+/CD4^-$. Nine of the clones were $CD4^+/CD8^-$, despite being specific for an HLA-A2 binding peptide. The remaining 5 clones contained varying levels of both CD4 and CD8. The majority (19/21) of clones expressed the α/β TCR, but interestingly, 2 clones expressed the γ/δ TCR. Several of these clones could be induced to secrete IFN-γ in response to p369-377 peptide stimulation. Several clones also lysed HLA-A2-transfected HER-2/neu-overexpressing tumor cells, including the γ/δ TCR-expressing clones. Investigations such as these demonstrate that *ex vivo* expansion and characterization of ovarian cancer-specific T cells is facilitated by prior vaccination. Prior vaccination and improved *ex vivo* expansion techniques lay the foundation for testing of adoptive T cell therapy in ovarian cancer patients.

Redirection of peripheral blood naive T cells to recognize ovarian cancer antigens is also being evaluated. In a current clinical trial (NCI 960011), advanced-stage ovarian cancer patients are being treated with escalating doses of peripheral blood lymphocytes that have been genetically modified to recognize FBP. The trial is based on extensive preclinical data evaluating the *in vitro* and *in vivo* efficacy of lymphocytes transduced with a chimeric receptor encoding the antigen recognition domain of MOv18 antibody fused to the Fc receptor γ chain [94, 95]. Initial studies tested the concept *in vitro* using tumor infiltrating lymphocytes (TIL) isolated from melanoma cancer patients [94]. Melanoma-specific TIL could be redirected, with the chimeric receptor, to lyse and secrete cytokine in response to FBP-overexpressing ovarian tumor cells. To test the *in vivo* efficacy, murine TIL were transduced and used in adoptive transfer experiments in nude mice implanted with human ovarian cancers. A single dose of 10 to 30 million transduced T cells greatly improved survival by limiting tumor growth. The ongoing clinical trial using this approach is testing the efficacy of intravenous infusion of retroviral-transduced peripheral blood lymphocytes that are activated with the anti-CD3 mAb, OKT3 prior to infusion.

Intraperitoneal delivery of cytokines has shown to be efficacious in some patients with advanced disease. The cytokines IFN-γ, IFN-α, or IL-2 have all been examined for efficacy in a number of clinical trials. IFN-γ potentiates the ip. immune response by upregulating the expression of MHC class I and class II on APC, T cells and tumor cells [96]. Ip. IFN-γ has produced complete responses in patients in whom previous chemotherapy had failed. In one study, ip. IFN-γ was given as second-line treatment in patients with persistent disease at second-look laparotomy [97]. Of 98 evaluable patients, 31 (32%) achieved a surgically documented response, including 23 patients (23%) with a complete response. The responses were generally better in younger patients (<60 years old) with smaller tumors (<2 cm). IFN-γ administration was very well-tolerated.

IFN-α also has been extensively tested in patients with advanced, refractory ovarian cancer. IFN-α has mechanisms of action similar to IFN-γ. Initial studies indicated that responses with ip. injection of IFN-α could be achieved in patients with aggressive tumors. For example, Berek and colleagues evaluated 14 patients with persistent ovarian disease following ip. treatment with 5×10^6 units IFN-α escalated over 4 weeks to 50×10^6 units [98]. The high dose was then continued for an additional 16 weeks. Of 11 patients who underwent surgical reevaluation, 4 had complete responses, one had a partial response, and disease progression was observed in 6 patients. If the patients were stratified according to tumor size, 5 of 7 patients with tumors of less than 5 mm demonstrated surgically documented responses, while no responses were observed in patients with tumors greater

than or equal to 5 mm. The responses were accompanied by increased ip. numbers of monocytes and lymphocytes. These encouraging results led to a phase II trial treating 92 patients with residual disease of less than 5 mm [99]. Forty-six evaluable patients were stratified either to a platinum-sensitive group of 25 or a platinum-resistant group of 21. None of the patients that demonstrated platinum resistance showed a clinical response. However, 4 (16%) of the platinum-sensitive group demonstrated complete responses, 3 (12%) had partial responses and 8 (32%) showed no response. The remaining 10 patients had progressive disease prior to planned surgical reevaluation. These results suggested that future trials of IFN-α should be directed at a subset of patients with advanced stage platinum-sensitive disease.

Intraperitoneal IL-2 has also been examined in patients with persistent disease following chemotherapy. Edwards and colleagues reported the results of a Phase I/II trial involving 45 patients [100]. Patients were treated with 1 of 4 doses of IL-2 (6×10^4, 6×10^5, 6×10^6, and 3×10^7 IU/m^2) given as either weekly 24-hour infusions or continuous 7 day infusions. Of 35 assessable patients, 6 had confirmed complete responses and 3 had partial responses. An assessment of survival revealed that patients with responses to IL-2 had prolonged survival. However, clinical trials enrolling larger numbers of patients will be required to discern any overall improvement in survival compared to those not treated with IL-2. On the whole, treatment with ip. cytokines appears to be effective in subsets of patients. Although complete clinical responses remain relatively low, stratification of patients may continue to improve our understanding of the mechanism of action of these agents and provide critical predictive insight into what subsets of resistant patients may respond to immune-based therapies. The development of modern genomic and proteomic techniques could elucidate the tumor characteristics that would predict a more favorable response to therapy. Alternatively, the use of cytokines may be better suited for patients with no evidence of disease or only microscopic disease. IL-2 is also being examined in combination with IFN-γ or adoptive T cell therapy [101, 102]. Strategies to reverse underlying tolerance or immunosuppression may improve efficacy when used in conjunction with these agents.

VII. Conclusions

Ovarian cancer is an immunogenic tumor and numerous antigens have been identified in recent years. Several of these antigens are important in regulating tumor growth and may be ideal targets for the development of immune-based strategies. In the absence of immunologic intervention, tumors evade the immune system by several

mechanisms, most notably tolerance and immunosuppression. As our understanding of the immune response improves, strategies are being designed to circumvent T cell tolerance to self antigens through modulation of APC function. In addition, techniques are being developed to identify reverse ovarian cancer-induced immune evasion tactics. The type of the immune-based therapy to apply will vary with disease burden. Hopefully discoveries at the bench along with lessons learned in prior clinical trials will soon allow us to develop rationally-based immunologic strategies to treat and prevent ovarian cancer.

VIII. Reference List.

1. Peoples G.E., Anderson B.W., Lee T.V., Murray J.L., Kudelka A.P., Wharton J.T., *et al.*, Vaccine implications of folate binding protein, a novel cytotoxic T lymphocyte-recognized antigen system in epithelial cancers. Clin Cancer Res, 1999;5(12):4214-23.

2. Li P.Y., Del Vecchio S., Fonti R., Carriero M.V., Potena M.I., Botti G., *et al.*, Local concentration of folate binding protein GP38 in sections of human ovarian carcinoma by in vitro quantitative autoradiography. J Nucl Med, 1996;37(4):665-72.

3. Bottero F., Tomassetti A., Canevari S., Miotti S., Menard S., and Colnaghi M.I., Gene transfection and expression of the ovarian carcinoma marker folate binding protein on NIH/3T3 cells increases cell growth in vitro and in vivo. Cancer Res, 1993;53(23):5791-6.

4. Samanta A., LeVea C.M., Dougall W.C., Qian X., and Greene M.I., Ligand and p185c-neu density govern receptor interactions and tyrosine kinase activation. Proc Natl Acad Sci U S A, 1994;91(5):1711-5.

5. Olayioye M.A., Neve R.M., Lane H.A., and Hynes N.E., The ErbB signaling network: receptor heterodimerization in development and cancer. Embo J, 2000;19(13):3159-67.

6. Slamon D.J., Godolphin W., Jones L.A., Holt J.A., Wong S.G., Keith D.E., *et al.*, Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science, 1989;244(4905):707-12.

7. Hellstrom I., Goodman G., Pullman J., Yang Y., and Hellstrom K.E., Overexpression of HER-2 in ovarian carcinomas. Cancer Res, 2001;61(6):2420-3.

8. Gillespie A.M., Rodgers S., Wilson A.P., Tidy J., Rees R.C., Coleman R.E., *et al.*, MAGE, BAGE and GAGE: tumour antigen expression in benign and malignant ovarian tissue. Br J Cancer, 1998;78(6):816-21.

9. Osterlund C., Tohonen V., Forslund K.O., and Nordqvist K., Mage-b4, a novel melanoma antigen (MAGE) gene specifically expressed during germ cell differentiation. Cancer Res, 2000;60(4):1054-61.

10. Yamada A., Kataoka A., Shichijo S., Kamura T., Imai Y., Nishida T., *et al.*, Expression of MAGE-1, MAGE-2, MAGE-3/-6 and MAGE-4a/-4b genes in ovarian tumors. Int J Cancer, 1995;64(6):388-93.

11. Gendler S.J. and Spicer A.P., Epithelial mucin genes. Annu Rev Physiol, 1995;57:607-34.

12. Brossart P., Schneider A., Dill P., Schammann T., Grunebach F., Wirths S., *et al.*, The epithelial tumor antigen MUC1 is expressed in hematological malignancies and is recognized by MUC1-specific cytotoxic T-lymphocytes. Cancer Res, 2001;61(18):6846-50.

13. Agrawal B., Krantz M.J., Reddish M.A., and Longenecker B.M., Rapid induction of primary human CD4+ and CD8+ T cell responses against cancer-associated MUC1 peptide epitopes. Int Immunol, 1998;10(12):1907-16.

14. Ioannides C.G., Fisk B., Jerome K.R., Irimura T., Wharton J.T., and Finn O.J., Cytotoxic T cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. J Immunol, 1993;151(7):3693-703.

15. Berchuck A., Kohler M.F., Marks J.R., Wiseman R., Boyd J., and Bast R.C., Jr., The p53 tumor suppressor gene frequently is altered in gynecologic cancers. Am J Obstet Gynecol, 1994;170(1 Pt 1):246-52.

16. Green JA M.B., Jenkins J, Leinster SJ, Tarunina M, Green B, Robertson L, Serum p53 auto-antibodies: incidence in familial breast cancer. Eur. J. Cancer, 1994;30A(5):580-584.

17. Angelopoulou K., Rosen B., Stratis M., Yu H., Solomou M., and Diamandis E.P., Circulating antibodies against p53 protein in patients with ovarian carcinoma. Correlation with clinicopathologic features and survival. Cancer, 1996;78(10):2146-52.

18. Wurtzen P.A. and Claesson M.H., A HLA-A2 restricted human CTL line recognizes a novel tumor cell expressed p53 epitope. Int J Cancer, 2002;99(4):568-72.

19. Hoffmann T.K., Donnenberg A.D., Finkelstein S.D., Donnenberg V.S., Friebe-Hoffmann U., Myers E.N., *et al.*, Frequencies of tetramer+ T cells specific for the wild-type sequence p53(264-272) peptide in the circulation of patients with head and neck cancer. Cancer Res, 2002;62(12):3521-9.

20. Kobayashi H., Terao T., and Kawashima Y., Serum sialyl Tn as an independent predictor of poor prognosis in patients with epithelial ovarian cancer. J Clin Oncol, 1992;10(1):95-101.

21. Federici M.F., Kudryashov V., Saigo P.E., Finstad C.L., and Lloyd K.O., Selection of carbohydrate antigens in human epithelial ovarian cancers as targets for immunotherapy: serous and mucinous tumors exhibit distinctive patterns of expression. Int J Cancer, 1999;81(2):193-8.

22. Taylor D.D., Bender D.P., Gercel-Taylor C., Stanson J., and Whiteside T.L., Modulation of TcR/CD3-zeta chain expression by a circulating factor derived from ovarian cancer patients. Br J Cancer, 2001;84(12):1624-9.

23. Zou W., Machelon V., Coulomb-L'Hermin A., Borvak J., Nome F., Isaeva T., *et al.*, Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells. Nat Med, 2001;7(12):1339-46.

24. Nagasawa T., Hirota S., Tachibana K., Takakura N., Nishikawa S., Kitamura Y., *et al.*, Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature, 1996;382(6592):635-8.

25. Nash M.A., Ferrandina G., Gordinier M., Loercher A., and Freedman R.S., The role of cytokines in both the normal and malignant ovary. Endocr Relat Cancer, 1999;6(1):93-107.

26. Gabrilovich D.I., Chen H.L., Girgis K.R., Cunningham H.T., Meny G.M., Nadaf S., *et al.*, Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. Nat Med, 1996;2(10):1096-103.

27. Shevach E.M., CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol, 2002;2(6):389-400.

28. Woo E.Y., Chu C.S., Goletz T.J., Schlienger K., Yeh H., Coukos G., *et al.*, Regulatory CD4(+)CD25(+) T cells in tumors from patients with early- stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res, 2001;61(12):4766-72.

29. Tempfer C., Hefler L., Haeusler G., Sliutz G., Hanzal E., Reinthaller A., *et al.*, Serum M3/M21 in ovarian cancer patients. Br J Cancer, 1997;76(10):1387-9.

30. Obata N.H., Tamakoshi K., Shibata K., Kikkawa F., and Tomoda Y., Effects of interleukin-6 on in vitro cell attachment, migration and invasion of human ovarian carcinoma. Anticancer Res, 1997;17(1A):337-42.

31. Kryczek I., Grybos M., Karabon L., Klimczak A., and Lange A., IL-6 production in ovarian carcinoma is associated with histiotype and biological characteristics of the tumour and influences local immunity. Br J Cancer, 2000;82(3):621-8.

32. Baldwin R.L., Tran H., and Karlan B.Y., Primary ovarian cancer cultures are resistant to Fas-mediated apoptosis. Gynecol Oncol., 1999;74:265-71.

33. Rabinowich H., Reichert T.E., Kashii Y., Gastman B.R., Bell M.C., and Whiteside T.L., Lymphocyte apoptosis induced by Fas ligand- expressing ovarian carcinoma cells. Implications for altered expression of T cell receptor in tumor-associated lymphocytes. J Clin Invest, 1998;101(11):2579-88.

34. Dong H., Strome S.E., Salomao D.R., Tamura H., Hirano F., Flies D.B., *et al.*, Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat Med, 2002;8(8):793-800.

35. Peoples G.E., Goedegebuure P.S., Smith R., Linehan D.C., Yoshino I., and Eberlein T.J., Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc Natl Acad Sci, USA, 1995;92:432-436.

36. Disis M.L., Knutson K.L., Schiffman K., Rinn K., and McNeel D.G., Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. Breast Cancer Res Treat, 2000;62(3):245-52.

37. Guy C.T., Webster M.A., Schaller M., Parsons T.J., Cardiff R.D., and Muller W.J., Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci U S A, 1992;89(22):10578-82.

38. Reilly R.T., Gottlieb M.B., Ercolini A.M., Machiels J.P., Kane C.E., Okoye F.I., *et al.*, HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. Cancer Res, 2000;60(13):3569-76.

39. Surman D.R., Dudley M.E., Overwijk W.W., and Restifo N.P., Cutting edge: CD4+ T cell control of CD8+ T cell reactivity to a model tumor antigen. J Immunol, 2000;164(2):562-5.

40. Hung K., Hayashi R., Lafond-Walker A., Lowenstein C., Pardoll D., and Levitsky H., The central role of CD4(+) T cells in the antitumor immune response. J Exp Med, 1998;188(12):2357-68.

41. Disis M.L., Grabstein K.H., Sleath P.R., and Cheever M.A., Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. Clin Cancer Res, 1999;5(6):1289-97.

42. Steinman R.M. and Dhodapkar M., Active immunization against cancer with dendritic cells: the near future. Int J Cancer, 2001;94(4):459-73.

43. Esche C., Cai Q., Peron J.M., Hunter O., Subbotin V.M., Lotze M.T., *et al.*, Interleukin-12 and Flt3 ligand differentially promote dendropoiesis in vivo. Eur J Immunol, 2000;30(9):2565-75.

44. Josien R., Li H.L., Ingulli E., Sarma S., Wong B.R., Vologodskaia M., *et al.*, TRANCE, a tumor necrosis factor family member, enhances the longevity and adjuvant properties of dendritic cells in vivo. J Exp Med, 2000;191(3):495-502.

45. Kim C.H., Pelus L.M., White J.R., Applebaum E., Johanson K., and Broxmeyer H.E., CK beta-11/macrophage inflammatory protein-3 beta/EBI1-ligand chemokine is an efficacious chemoattractant for T and B cells. J Immunol, 1998;160(5):2418-24.

46. Fiebiger E., Meraner P., Weber E., Fang I.F., Stingl G., Ploegh H., *et al.*, Cytokines regulate proteolysis in major histocompatibility complex class ii-dependent antigen presentation by dendritic cells. J Exp Med, 2001;193(8):881-92.

47. Lynch D.H., Andreasen A., Maraskovsky E., Whitmore J., Miller R.E., and Schuh J.C., Flt3 ligand induces tumor regression and antitumor immune responses in vivo. Nat Med, 1997;3(6):625-31.

48. Brunda M.J., Luistro L., Warrier R.R., Wright R.B., Hubbard B.R., Murphy M., *et al.*, Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J Exp Med, 1993;178(4):1223-30.

49. Van Niekerk C.C., Ramaekers F.C., Hanselaar A.G., Aldeweireldt J., and Poels L.G., Changes in expression of differentiation markers between normal ovarian cells and derived tumors. Am J Pathol, 1993;142(1):157-77.

50. Moretta L., Bottino C., Pende D., Mingari M.C., Biassoni R., and Moretta A., Human natural killer cells: their origin, receptors and function. Eur J Immunol, 2002;32(5):1205-11.

51. Silver D.F., Hempling R.E., Piver M.S., and Repasky E.A., Effects of IL-12 on human ovarian tumors engrafted into SCID mice. Gynecol Oncol, 1999;72(2):154-60.

52. Shi F., Ljunggren H.G., and Sarvetnick N., Innate immunity and autoimmunity: from self-protection to self- destruction. Trends Immunol, 2001;22(2):97-101.

53. Nishimura T., Kitamura H., Iwakabe K., Yahata T., Ohta A., Sato M., *et al.*, The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. Int Immunol, 2000;12(7):987-94.

54. Baxevanis C.N., Gritzapis A.D., Tsitsilonis O.E., Katsoulas H.L., and Papamichail M., HER-2/neu-derived peptide epitopes are also recognized by cytotoxic CD3(+)CD56(+) (natural killer T) lymphocytes. Int J Cancer, 2002;98(6):864-72.

55. Dyatlovitskaya E.V., Andreasyan G.O., Malykh Ya N., Rylova S.N., and Somova O.G., Ganglioside shedding and changes in ceramide biosynthesis in human ovarian tumors. Biochemistry (Mosc), 1997;62(5):557-61.

56. Soper J.T., Johnson P., Johnson V., Berchuck A., and Clarke-Pearson D.L., Comprehensive restaging laparotomy in women with apparent early ovarian carcinoma. Obstet Gynecol, 1992;80(6):949-53.

57. Cheever M.A. and Chen W., Therapy with cultured T cells: principles revisited. Immunol Rev, 1997;157:177-94.

58. Drake C.G. and Pardoll D.M., Tumor immunology--towards a paradigm of reciprocal research. Semin Cancer Biol, 2002;12(1):73-80.

59. Perales M.A., Blachere N.E., Engelhorn M.E., Ferrone C.R., Gold J.S., Gregor P.D., *et al.*, Strategies to overcome immune ignorance and tolerance. Semin Cancer Biol, 2002;12(1):63-71.

60. Banchereau J., Schuler-Thurner B., Palucka A.K., and Schuler G., Dendritic cells as vectors for therapy. Cell, 2001;106(3):271-4.

61. Brossart P., Wirths S., Stuhler G., Reichardt V.L., Kanz L., and Brugger W., Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. Blood, 2000;96(9):3102-8.

62. Hernando J.J., Park T.W., Kubler K., Offergeld R., Schlebusch H., and Bauknecht T., Vaccination with autologous tumour antigen-pulsed dendritic cells in advanced gynaecological malignancies: clinical and immunological evaluation of a phase I trial. Cancer Immunol Immunother, 2002;51(1):45-52.

63. Disis M.L., Bernhard H., Shiota F.M., Hand S.L., Gralow J.R., Huseby E.S., *et al.*, Granulocyte-macrophage colony-stimulating factor: an effective adjuvant for protein and peptide-based vaccines. Blood, 1996;88(1):202-210.

64. Disis M.L., Gooley T.A., Rinn K., Davis D., Piepkorn M., Cheever M.A., *et al.*, Generation of T-cell immunity to the her-2/neu protein after active immunization with her-2/neu Peptide-based vaccines. J Clin Oncol, 2002;20(11):2624-32.

65. Holmberg L.A., Oparin D.V., Gooley T., Lilleby K., Bensinger W., Reddish M.A., *et al.*, Clinical outcome of breast and ovarian cancer patients treated with high-dose chemotherapy, autologous stem cell rescue and THERATOPE STn- KLH cancer vaccine. Bone Marrow Transplant, 2000;25(12):1233-41.

66. Riker A., Cormier J., Panelli M., Kammula U., Wang E., Abati A., *et al.*, Immune selection after antigen-specific immunotherapy of melanoma. Surgery, 1999;126(2):112-20.

67. Jager E., Ringhoffer M., Altmannsberger M., Arand M., Karbach J., Jager D., *et al.*, Immunoselection in vivo: independent loss of MHC class I and melanocyte differentiation antigen expression in metastatic melanoma. Int J Cancer, 1997;71(2):142-7.

68. Vanderlugt C.J. and Miller S.D., Epitope spreading. Curr Opin Immunol, 1996;8(6):831-6.

69. Ward S., Casey D., Labarthe M.C., Whelan M., Dalgleish A., Pandha H., *et al.*, Immunotherapeutic potential of whole tumour cells. Cancer Immunol Immunother, 2002;51(7):351-7.

70. Ada G., The coming of age of tumour immunotherapy. Immunol Cell Biol, 1999;77(2):180-5.

71. Agrawal B., Krantz M.J., Reddish M.A., and Longenecker B.M., Cancer-associated MUC1 mucin inhibits human T-cell proliferation, which is reversible by IL-2. Nat Med, 1998;4(1):43-9.

72. Jager D., Jager E., and Knuth A., Immune responses to tumour antigens: implications for antigen specific immunotherapy of cancer. J Clin Pathol, 2001;54(9):669-74.

73. Baselga J., Clinical trials of Herceptin(R) (trastuzumab). Eur J Cancer, 2001;37 Suppl 1:18-24.

74. King K.M. and Younes A., Rituximab: review and clinical applications focusing on non-Hodgkin's lymphoma. Expert Rev Anticancer Ther, 2001;1(2):177-86.

75. Carter P., Improving the efficacy of antibody-based cancer therapies. Nat Rev Cancer, 2001;1(2):118-29.

76. Cragg M.S., French R.R., and Glennie M.J., Signaling antibodies in cancer therapy. Curr Opin Immunol, 1999;11(5):541-7.

77. Baselga J., Albanell J., Molina M.A., and Arribas J., Mechanism of action of trastuzumab and scientific update. Semin Oncol, 2001;28(5, Suppl 16):4-11.

78. Sondel P.M. and Hank J.A., Antibody-directed, effector cell-mediated tumor destruction. Hematol Oncol Clin North Am, 2001;15(4):703-21.

79. zum Buschenfelde C.M., Hermann C., Schmidt B., Peschel C., and Bernhard H., Antihuman epidermal growth factor receptor 2 (HER2) monoclonal antibody trastuzumab enhances cytolytic activity of class I-restricted HER2- specific T lymphocytes against HER2-overexpressing tumor cells. Cancer Res, 2002;62(8):2244-7.

80. Alvarez R.D., Barnes M.N., Gomez-Navarro J., Wang M., Strong T.V., Arafat W., *et al.*, A cancer gene therapy approach utilizing an anti-erbB-2 single-chain antibody-encoding adenovirus (AD21): a phase I trial. Clin Cancer Res, 2000;6(8):3081-7.

81. Chen H.X., Gore-Langton R.E., and Cheson B.D., Clinical trials referral resource: Current clinical trials of the anti- VEGF monoclonal antibody bevacizumab. Oncology (Huntingt), 2001;15(8):1017, 1020, 1023-6.

82. Sledge G.W., Jr., Vascular endothelial growth factor in breast cancer: biologic and therapeutic aspects. Semin Oncol, 2002;29(3 Suppl 11):104-10.

83. Presta L.G., Chen H., O'Connor S.J., Chisholm V., Meng Y.G., Krummen L., *et al.*, Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res, 1997;57(20):4593-9.

84. Johnson D.H., DeVore R., Kabbinavar F., Herbst R., Holmgren E., and Novotny W., Carboplatin (C) + Paclitaxel (T) + RhuMab-VEGF (AVF) may prolong survival in advanced non-squamous lung cancer. Proc. Amer. Soc. Clin. Onc., 2001;20:A315.

85. Toffoli G., Cernigoi C., Russo A., Gallo A., Bagnoli M., and Boiocchi M., Overexpression of folate binding protein in ovarian cancers. Int J Cancer, 1997;74(2):193-8.

86. Coney L.R., Tomassetti A., Carayannopoulos L., Frasca V., Kamen B.A., Colnaghi M.I., *et al.*, Cloning of a tumor-associated antigen: MOv18 and MOv19 antibodies recognize a folate-binding protein. Cancer Res, 1991;51(22):6125-32.

87. Crippa F., Bolis G., Seregni E., Gavoni N., Scarfone G., Ferraris C., *et al.*, Single-dose intraperitoneal radioimmunotherapy with the murine monoclonal antibody I-131 MOv18: clinical results in patients with minimal residual disease of ovarian cancer. Eur J Cancer, 1995;5:686-90.

88. Van Zanten-Przybysz I., Molthoff C., Gebbinck J.K., Von Mensdorff-Pouilly S., Verstraeten R., Kenemans P., *et al.*, Cellular and humoral responses after multiple injections of unconjugated chimeric monoclonal antibody MOv18 in ovarian cancer patients: a pilot study. J Cancer Res Clin Oncol, 2002;128(9):484-92.

89. van Zanten-Przybysz I., Molthoff C.F., Roos J.C., Plaizier M.A., Visser G.W., Pijpers R., *et al.*, Radioimmunotherapy with intravenously administered 131I-labeled chimeric monoclonal antibody MOv18 in patients with ovarian cancer. J Nucl Med, 2000;41(7):1168-76.

90. Knutson K.L., Almand B., Mankoff D.A., Schiffman K., and Disis M.L., Adoptive T-cell therapy for the treatment of solid tumours. Expert Opin Biol Ther, 2002;2(1):55-66.

91. Dudley M.E., Wunderlich J.R., Robbins P.F., Yang J.C., Hwu P., Schwartzentruber D.J., *et al.*, Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science, 2002;298(5594):850-4.

92. Knutson K.L. and Disis M.L., Expansion of HER-2/neu specific T cells *ex vivo* following immunization with a HER-2/neu peptide-based vaccine. Clin Breast Ca, 2001;2:73-79.

93. Knutson K.L., Schiffman K., Cheever M.A., and Disis M.L., Immunization of cancer patients with a HER-2/neu, HLA-A2 peptide, p369- 377, results in short-lived peptide-specific immunity. Clin Cancer Res, 2002;8(5):1014-8.

94. Hwu P., Shafer G.E., Treisman J., Schindler D.G., Gross G., Cowherd R., *et al.*, Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor gamma chain. J Exp Med, 1993;178(1):361-6.

95. Hwu P., Yang J.C., Cowherd R., Treisman J., Shafer G.E., Eshhar Z., *et al.*, In vivo antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes. Cancer Res, 1995;55(15):3369-73.

96. Allavena P., Peccatori F., Maggioni D., Erroi A., Sironi M., Colombo N., *et al.*, Intraperitoneal recombinant gamma-interferon in patients with recurrent ascitic ovarian carcinoma: modulation of cytotoxicity and cytokine production in tumor-associated effectors and of major histocompatibility antigen expression on tumor cells. Cancer Res, 1990;50(22):7318-23.

97. Pujade-Lauraine E., Guastalla J.P., Colombo N., Devillier P., Francois E., Fumoleau P., *et al.*, Intraperitoneal recombinant interferon gamma in ovarian cancer patients with residual disease at second-look laparotomy. J Clin Oncol, 1996;14(2):343-50.

98. Berek J.S., Hacker N.F., Lichtenstein A., Jung T., Spina C., Knox R.M., *et al.*, Intraperitoneal recombinant alpha-interferon for "salvage" immunotherapy in stage III epithelial ovarian cancer: a Gynecologic Oncology Group Study. Cancer Res, 1985;45(9):4447-53.

99. Berek J.S., Markman M., Blessing J.A., Kucera P.R., Nelson B.E., Anderson B., *et al.*, Intraperitoneal alpha-interferon alternating with cisplatin in residual ovarian carcinoma: a phase II Gynecologic Oncology Group study. Gynecol Oncol, 1999;74(1):48-52.

100. Edwards R.P., Gooding W., Lembersky B.C., Colonello K., Hammond R., Paradise C., *et al.*, Comparison of toxicity and survival following intraperitoneal recombinant interleukin-2 for persistent ovarian cancer after platinum: twenty-four-hour versus 7-day infusion. J Clin Oncol, 1997;15(11):3399-407.

101. Freedman R.S. and Platsoucas C.D., Immunotherapy for peritoneal ovarian carcinoma metastasis using ex vivo expanded tumor infiltrating lymphocytes. Cancer Treat Res, 1996;82:115-46.

102. Freedman R.S., Lenzi R., Kudelka A.P., Lawrence D.D., Rosenblum M., and Platsoucas C.D., Intraperitoneal immunotherapy of peritoneal carcinomatosis. Cytokines Cell Mol Ther, 1998;4(2):121-40.

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CHAPTER 17

Immunotherapy for breast cancer

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1. Introduction

Immune-based therapies for the treatment of breast cancer are now being designed to circumvent specific biologic problems that have been identified in the native immune response to breast cancer. Advances in basic immunology over the past decade have changed the scope and application of breast cancer immunotherapy. Perhaps the most important advances are the understanding that human tumors are immunogenic and the identification of tumor-specific antigens. In fact, hundreds of immunogenic proteins have been identified in patients with cancer using high throughput technologies such as serological identification of antigens by recombinant expression cloning (SEREX). More importantly, we have begun to identify proteins that are immunogenic in cancer whose function may be a causal component in the malignant transformation. Thus, immune-based therapies targeting these biologically relevant antigens may result in the eradication of tumor cell clones that drive the initiation of malignancy.

The roles of the immune and tumor microenvironments in breast cancer greatly influence our approach to the design of immunotherapies. Stimulation of both cytotoxic T cell (CTL) and CD4 'helper' T cell immunity is critically important for an effective antitumor response. In addition, cells of the innate immune system such as natural killer (NK) cells are likely to play an important role in a balanced antitumor response. Cytokines and chemokines can both inhibit and stimulate immune responses and tumor growth. Moreover, the identification of the dendritic cell (DC) and the understanding of its role in initiating immunity to cancer has not only led to novel therapies for the treatment of breast cancer, but has focused attention on the major role of the antigen-presenting cell (APC) in initiation or dampening of the tumor-specific immune response. We now know that the tumor microenvironment is dynamic. Molecular dissection of the evolution of the immune response occurring against autologous tumors in patients with advanced stage melanoma has demonstrated that the immune system is quite functional even in patients with significant existing tumor burden. In fact, stimulating immunity in patients with measurable disease may have beneficial effects in immune augmentation. Apoptosis or necrosis of tumor cells may be an efficient method of antigen presentation for initiation of immune responses. Likewise, the presence of antigen during the evolution of the immune response may be instrumental in generating T cell clonal expansion and epitope spreading, highlighting the occurrence of natural antigen-processing and the generation of endogenous tumor-specific immunity.

Finally, immune-based therapies are being applied clinically in different ways, both for treat-

ment and prevention. Vaccines targeting tumor antigens are increasingly being viewed and studied as chemopreventive agents designed to protect against cancer relapse or development. Established disease is being treated with immune-based therapies, such as adoptive immunotherapy or infusion of T cells allogeneic to host tumor cells therapies, designed to maximize T cell precursor frequencies. Thus, investigations of the immune system at both the bench and bedside have led to the development of rationally based immunotherapies for the prevention and treatment of breast cancer. Therapeutic approaches are now targeted to specific alterations in the evolution of the breast cancer-specific immune response.

2. Breast cancer tumor antigens

Recent studies by several groups have identified 'self' antigens, expressed on tumor cells, as tumor antigens [1,2]. These proteins are not mutated in any way, but are clearly immunogenic in patients with cancer and have been shown to generate both antibody and CTL responses in humans [1,3,4]. Many of these proteins are present at much higher concentrations in malignant cells than in the normal cells with which they are associated [5]. Gene amplification results in overexpression of normal cellular proteins in cancer and is an etiologic factor in the malignant transformation of many solid tumors. Overexpressed oncoproteins are not mutated and are distinct from their normal counterparts only by virtue of their greater concentration in cancer cells. Intuitively, these proteins would not be considered potential tumor antigens as patients should be tolerant to self-proteins. The recent finding, that many tumor antigens are self-proteins, has resulted in a 'paradigm shift' [6]. The new paradigm includes self-proteins as tumor antigens and tolerance induction as a possible mechanism of immune escape.

A multitude of self-tumor antigens are present in breast cancer including, CEA, MUC-1, a variety of glycoproteins, p53, and MAGE-3 to name a few. Of interest is the identification of immunogenic proteins in breast cancer that are also important cell regulatory proteins. By targeting the immune system to proteins that either initiate or propagate the malignant phenotype, immune-based therapies have the potential to eradicate the malignant clone. An example of two defined tumor antigens that are involved in the carcinogenic pathway of breast cancer are the HER-2/neu oncogenic protein and telomerase.

One of the most extensively described tumor antigens in breast cancer is the HER-2/neu oncogenic protein. HER-2/neu is a member of the epidermal growth factor receptor family and is a growth factor receptor [7,8]. HER-2/neu is a transmembrane protein that consists of a cysteine-rich extracellular domain that functions in ligand binding and a cytoplasmic domain with kinase activity. In humans, the HER-2/neu protein is expressed during fetal development [9]. In adults, the protein is weakly detectable in the epithelial cells of many normal tissues by immunohistochemical staining. The HER-2/neu gene is present in normal cells as a single copy [9]. Amplification of the gene and/or overexpression of the associated protein have been identified in many human cancers such as breast, ovarian, prostate, non-small cell lung cancer, and colon cancer and is associated with a poor prognosis. The HER-2/neu oncogenic protein is also a tumor antigen. Patients with different tumor types that overexpress the HER-2/neu protein can have both antibody [10] and T cell immunity directed against HER-2/neu [11]. Existent immunity to HER-2/neu in humans was initially described in patients with breast cancer [3].

Telomerase is a ribonucleoprotein enzyme. Normally telomerase maintains the telomeric ends of chromosomes protecting them from degradation. Non-malignant cells do not express telomerase, but malignant cells have increased telomerase activity presumably resulting in immortalization of the cell. The telomeric catalytic subunit (hTERT) is the rate-limiting component of the enzyme, and expression of hTERT correlates best with telomerase activity [12]. Although hTERT is

a self-protein, CTL specific for peptides derived from hTERT can lyse hTERT, expressing tumor cells in an HLA-A2-restricted fashion. The ability to recognize hTERT is within the realm of the T cell repertoire [12]. The definition of biologically important and ubiquitous proteins as tumor antigens in breast cancer alters our concept of what characteristics of a protein make it immunogenic. No longer are tumor antigens defined by unique mutations. The problem currently facing tumor immunologists is how to harness immunity to 'self' for cancer therapeutics. Both the immune and tumor microenvironments play an important role in regulating tumor-specific immunity.

3. Role of the immune microenvironment in regulating breast cancer immunity

Elucidation of breast cancer-specific immunity in animal models has demonstrated that an effective antitumor immune response will likely consist of complex interactions among multiple cellular and soluble immune effectors. Effector cells other than CTL that may induce an antitumor immune response include $CD4^+$ T cells, NK cells, NKT cells, DC, and other 'non-professional' APC such as macrophages. Furthermore, many chemokines and cytokines have been characterized that can have profound effects on immune effector cells as well as tumor cells.

CD8 or CTL have been thought to be major mediators of antitumor immunity because they exhibit direct killing of tumor cells in an MHC class I-restricted fashion. In studies in breast cancer patients, CTL established in vitro demonstrate significant levels of cytolytic activity against known breast cancer antigens such as MUC-1 and HER-2/neu [4,13]. In fact, similar to analyses performed in melanoma, recent studies evaluating the presence of class I molecules in breast cancer, have shown that class I can be down-regulated presumably due to immunoselection [14]. Therefore, CTL specific for breast cancer antigens do exist naturally in vivo in patients with breast cancer and may have a biologic effect on the tumor, but clearly are not sufficient in eradicating cancer. One problem with the pre-existent CTL response may be a lack of magnitude. An evaluation of immunity against the HER-2/neu antigen in patients with advanced stage breast cancer suggests that CTL precursor frequencies elicited by exposure to tumor are low, less than 1 : 100,000 of circulating T cells [15]. Direct modulation of the CTL immune effector arm is thought to be key to a successful antitumor response. However, recent developments suggest that CTL alone cannot constitute a complete antitumor response and that additional immune effectors such as $CD4^+$ T cells are needed for completing the appropriate immune milieu.

The $CD4^+$ T cell is critical in controlling the activation and persistence of the immune response against viral infections [16]. The interplay between $CD8^+$ and $CD4^+$ T cells in eradicating breast cancer has recently been evaluated in the neu transgenic (neuTg) mouse. neuTg mice are transgenic for rat neu under control of the MMTV promoter and develop breast cancers similar to those that occur in humans [17]. Also, as in humans, expression as a transgene confers immunologic tolerance to neu. The murine cancers generally start as hyperplasia, progress to ductal carcinomas in situ, and finally to frank interductal carcinomas [17]. Biologically relevant animal models, such as this one, can significantly aid in the definition of the functional components of the breast cancer-specific immune response as well as identify methods of overcoming tolerance. Although tolerant, neuTg mice can be vaccinated against neu and develop both neu-specific T cells and neu-specific IgG [18]. The resulting immune response, after active immunization, can delay the onset of the spontaneous neu-mediated tumors. In addition, depletion studies demonstrated that not only are $CD8^+$ T cells required for therapeutic efficacy, but also $CD4^+$ T cells. In fact, $CD4^+$ T cell-depleted mice showed faster tumor development than $CD8^+$-depleted mice, suggesting a dominant role for neu-specific $CD4^+$ T cells in eradicating the murine breast cancers.

Furthermore, to eradicate neu transgenic tumors implanted in SCID mice, both an infusion of neu-specific CTL and neu-specific IgG were needed [19]. The importance of $CD4^+$ T cells in the evolution of the tumor-specific immune response is underscored by recent studies demonstrating that $CD4^+$ T cells can initiate a de novo CTL response. Infusion of a tumor antigen-specific Th1 $CD4^+$ T cell clone resulted in the development of a $CD8^+$ antitumor immune response, presumably due to $CD4^+$ T cells secreting cytokines appropriate to enhance the function of local APC to cross-present tumor antigen to endogenous naïve $CD8^+$ T cells [20]. Once a $CD4^+$ T cell becomes activated a number of important events take place to influence the immune microenvironment. In addition to initiation of a CTL response, $CD4^+$ T cells can also produce cytokines that attract DC, macrophages, and eosinophils to promote an inflammatory environment [21]. As a result of establishing the necessity of $CD4^+$ T cell help in the immune response, much interest has focused on the identification of MHC class II binding epitopes of well-known human breast tumor antigens such as HER-2/neu [22] and NY-ESO-1 [23,24].

Although the antigen-specific T lymphocyte immune response is most often the focus of experimental and clinical investigation, cellular effectors of the innate immune system, such as NK and NKT cells also can induce an antitumor response. NK cells can kill tumors through a non-MHC-restricted mechanism. Newly defined NKT cells can specifically lyse tumor cells bearing glycolipid antigens. Inflammatory mediators, such as IL-12, may exert their antitumor effects through activation of NK cells. A murine model of metastatic breast cancer was developed to evaluate the therapeutic efficacy of administration of IL-12 in an adenoviral vector directly into liver lesions. Lesions regressed with treatment in almost all animals and complete tumor rejection occurred in 40% [25]. Cell subset depletion studies revealed that the antitumor effect seen with IL-12 was mediated significantly by NK cells. Although NK cells can possess direct antitumor cytolytic activity in vitro the role for the NK cell in mediating tumor rejection in vivo is still unclear. In addition to their ability to directly kill tumor, NK cells can also release a variety of type I cytokines such as IFNγ and TNFα which influence development of the adaptive immune response.

A newly identified cell of a novel lymphoid lineage, the Vα14 NKT cell, introduces an additional effector cell that may be important in an antitumor immune response. The NKT cell expresses typical NK cell surface receptors and a semi-invariant T cell receptor (TCR) encoded by Vα14 and Jα281 [26]. The NKT cell is activated through recognition of glycolipid antigens in association with CD1b, a MHC-like molecule present on APC. NKT cells secrete predominantly IL-4 and IFNγ and may be important in regulating the Th1/Th2 cytokine phenotype during an immune response [26]. Recently, Nishimura and colleagues have shown that $CD1d^+$ DC presentation of glycolipids to NKT cells can result in the generation of lymphoma-specific CTL, suggesting an important interface between the innate and adaptive antitumor immune response [27]. Glycolipids, such as ceramides and gangliosides are some of the most commonly studied breast cancer antigens [28,29]. Indeed, glycosylation of glycoproteins and glycolipids is a molecular change that often accompanies the malignant transformation of a breast cell [30]. In addition, $CD1^+$ DC are often observed in breast cancer biopsies [30]. Therapeutic infusion of NKT cells may be uniquely suited for the treatment of breast cancer.

The cellular immune response can not be elicited without effective APC processing and presentation of tumor proteins to T cells. DC are potent APC and are important in initiating a T cell response. DC usually exist in peripheral tissue in an immature state awaiting encounter with antigen. Immature DC are proficient at antigen uptake and processing but have no capacity to activate T cells. Following exposure to antigen, DC migrate to regional draining lymph nodes (DLN) and it is during migration that DC at-

tain the ability to present antigen, i.e. undergo maturation. Antigen-specific T lymphocytes are then stimulated by direct contact with the DC. Although not well described for breast cancer, most tumors fail to express MHC class II and are, therefore, not direct targets of $CD4^+$ T cells. The tumor cells would be immunogenic by other means such as uptake of the tumor cell debris by APC and 'cross-priming' [31]. The uptake, processing, and presentation of exogenously derived tumor antigens by DC, therefore, is important for activation of $CD4^+$ T cells. DC can acquire antigen by multiple means including endocytosis of soluble protein and phagocytosis of tumor-derived exosomes and apoptotic bodies [32,33]. Recent investigations have demonstrated that DC are associated with breast cancer tissue. Studies by Bell and colleagues have revealed that the maturation state of the DC differs with proximity to the breast cancer lesion [34]. Immature DC reside within the tumors, while mature DC are located in peritumoral areas. The intratumoral localization of immature DC suggests that apoptosis-inducing or tumor necrotizing agents may be effective in treating breast cancer as this may result in the cross-presentation of tumor-specific antigen to $CD4^+$ T cells. Indeed, Candido and colleagues have recently observed that the level of tumor cell apoptosis is positively correlated to the effectiveness of DC-based therapy of murine MT-901 breast carcinomas [35].

Cytokines released by both T cells and other cells involved in the inflammatory response greatly influence the immune microenvironment. Traditionally, T cell responses are categorized into a type I, e.g. TNFα-, IFNγ-secreting or type II, e.g. IL-4-, IL-10- secreting phenotype. Type I cytokine T cell responses are typically associated with the development of cell-mediated immune response while type II cytokines lead to the development of a humoral response and down-modulation of CTL. The interaction between cytokines and the stimulation of a T cell response is complex. As an example, early studies demonstrating that IFNγ could activate CTL, macrophages, and NK cells as well as up-regulate the level of MHC on a variety of immune effectors suggested that this cytokine is a major mediator of the antitumor immune response [36–39]. More recent investigations in $IFN\gamma^{-/-}$ mice, however, demonstrate that the function of IFNγ in mediating an antitumor immune response is complex. $IFN\gamma^{-/-}$ mice were immunized with syngeneic tumor cells. Spleen cells from immunized mice demonstrated antitumor activity, but when mice were challenged with viable tumor, they were not protected. T cell infiltration of tumor did not occur in the $IFN\gamma^{-/-}$ mice demonstrating that IFNγ was necessary for migration of both $CD4^+$ and CTL to tumor sites [40]. Thus, a major influence of IFNγ at the tumor site may be as a chemoattractant.

NK cells are also targets of cytokines and chemokines as recently described in murine breast cancer models. It is well known that IL-12 can directly mediate tumor killing. Many mechanisms have been described for the effects of IL-12 including increasing the local production of IFNγ and the inhibition of angiogenesis [41]. IL-12 is also known to directly stimulate NK cells [25]. NK cells can also be activated by the chemokine CKβ-11 to kill breast cancer cells [42]. CKβ-11 functions as a chemoattractant for many immune effector cells including T cells, B cells, DC, and NK cells [43–45]. In a study by Braun and colleagues, CKβ-11 was transfected and expressed intratumorally in murine breast cancer. The resultant tumor rejection involved both NK and T lymphocytes.

Recent studies have focused on the in vivo modulation of APC function by cytokines which can act at many different levels including APC differentiation [46], lifespan [47], migration [43], and antigen presentation [48]. Several cytokines are known to promote dendropoiesis, including IL-12 and Flt3 ligand, both of which have been shown to mediate tumor regression in vivo [49,50]. While the administration of either IL-12 or Flt-3 ligand increases the total number of circulating DC, IL-12 promotes myeloid den-

dropoiesis exclusively, while Flt3 ligand promotes both myeloid and lymphoid dendropoiesis [46]. In a study by Esche and colleagues, the addition of both IL-12 and Flt3 ligand synergized the expression of MHC class II, suggesting that antigen presentation may be enhanced with combination cytokine treatment. Increasing the numbers of DC in the draining lymph nodes can potentiate immune responses. DC die rapidly upon migration to the DLN, limiting the time of antigen exposure and the resulting immune response. A newly discovered TNF-related cytokine, TNF-related activation-induced cytokine (TRANCE), has been shown to enhance the lifespan of ex vivo manipulated DC [47]. By increasing the lifespan of antigen-presenting DC in the DLN, T cell responses to antigen were substantially increased [47]. Cytokines can down-regulate immune responses as well. As an example, lysosomal proteases of the cathepsin family are involved in processing of antigen in the context of MHC class II. IFNγ modulates cathepsin activity in cells [51]. Fieberger and colleagues recently examined the effects of proinflammatory and anti-inflammatory cytokines on the activities of cathepsin S and cathepsin B in human DC [48]. Treatment of DC with the proinflammatory cytokines TNFα and IL-1β leads to a rapid increase in the levels of DC cathepsin while the anti-inflammatory cytokine IL-10 renders the DC incapable of up-regulation of cathepsin. Coincidentally, IL-10 also reduces the number of MHC class II antigen complexes on the cell surface. Thus, cytokines can function to enhance or dampen the breast cancer-specific immune response in vivo.

4. Role of the tumor microenvironment in regulating breast cancer immunity

Human tumors can suppress the development of an immune response. However, even in advanced stage cancer, immunosuppression in patients may not be complete. In addition to the remarkable plasticity and ability to change phenotype, tumors can evade immune recognition by several mechanisms including tolerance, ignorance, inducing T cell dysfunction, and T cell death.

Tumor-specific immunity developing in a patient with advanced stage cancer is well documented. Coulie and colleagues recently published a 15-year chronology of immunologic studies of a melanoma patient with multiple recurrences but with a favorable clinical evolution due to immunologic intervention, both natural and pharmacological [52]. The findings provided key in vivo evidence that the generation of an immune response can potentially result in the breakthrough of unrecognized variant tumor cells. The patient initially presented with a primary melanoma that was surgically removed. Following three relapses, a cell line, MEL A by the patient, was established from a subcutaneous metastases and the patient underwent repeated vaccinations with the cell line and was disease-free for 4 years. This disease-free interval was associated with strong antitumor CTL response. CTL clones isolated from peripheral blood revealed several antigens produced by MEL A cells could be recognized in association with several HLA alleles. Eventually the patient relapsed with a tumor that had lost a complete HLA haplotype (A28-B44-Cw7). However, the original antigens were still expressed on the tumor. All of the CTL clones that were isolated before the relapse were A28- and B44-restricted. The development of escape variants has yet to be described in breast cancer, but as antigen-specific immune-based therapies are clinically tested evaluations of both antigen and immunostimulatory molecules on relapsed disease should be performed. Studies such as this one demonstrate that cancer patients can develop functional immune responses but that tumors can evade restricted immune responses.

Tolerance to self-tumor antigens is a major obstacle to overcome in the development of significant tumor-specific immunity. Tolerance can be mediated centrally and peripherally. Central tolerance occurs when antigens are expressed intrathymically resulting in deletion of the self-reactive T cells during early T cell differentiation

[53]. Central tolerance is thought to delete primarily high-affinity T cells that recognize dominant peptide epitopes, while lower-affinity T cells and those recognizing subdominant epitopes are allowed to escape into the periphery. Central tolerance leads to irreversible loss of autoantigen-reactive T cells by inducing apoptosis, and it is only those autoreactive T cells that escape thymic deletion that are of importance clinically. The T cells that avoid thymic clearance, however, are often tolerant or ignorant of tissues expressing the target antigens. As tumor antigen-specific T cells can be found in patients with cancer, it is clear that the ability to recognize cancer antigens is within the realm of the T cell repertoire and that central tolerance is not complete. Peripheral tolerance occurs extrathymically and is mediated by both deletional and non-deletional mechanisms [54]. The T cells that have been tolerized by non-deletional methods are the focus of immune-based antitumor therapies. A major non-deletional mechanism of peripheral tolerance is anergy induction. Anergy induction of antigen-specific T cells occurs by multiple mechanisms. The best understood mechanism of T cell anergy induction is TCR signaling in the absence of co-stimulation. Activation of T cells is thought to involve at least two independent signals, one mediated through the TCR : MHC complex and another through any of a variety of co-stimulatory receptors on T cells including CD28. Tumor cells, including breast cancer, do not typically express receptors (e.g. CD80) to these co-stimulatory molecules and may be responsible for anergy induction. In fact, recent reports demonstrate that even APC in the peripheral blood of early stage breast cancer patients demonstrate decreased expression of CD80 and CD86 [55]. Anergic T cells when rechallenged with peptide-pulsed APC fail to proliferate and have reduced cytokine release. Indeed, transfection of CD80 into many tumors, including breast cancer, can restore immune recognition and cause growth arrest [56].

The recovery of anergic T cells and the reversal of tolerance is critical to successful development of immune-based strategies for the treatment of breast cancer. Although it is generally presumed that self-reactive T cells are low-affinity by virtue of tolerizing processes, recent evidence by Ohlen and colleagues suggest that higher-affinity peripherally tolerized tumor-antigen-specific T cells can be recovered following multiple rounds of in vitro stimulation. Studies demonstrated that gag-specific T cells could be isolated from mice transgenically engineered to express the Friend murine leukemia virus protein, gag, within hepatocytes [57]. The gag-specific T cells, after several in vitro stimulations with repeated IL-2 administration, displayed CTL activity and were comparable in affinity to the gag-specific T cells isolated from the parental mouse, C57BL/6, in which gag is a foreign antigen. These findings suggest the potential for circumvention of tolerance and the recovery of high-affinity tumor-specific T cells using ex vivo techniques.

Cytokines present within the tumor microenvironment can also promote T cell anergy. The immunoregulatory cytokine, IL-10, has previously been shown to induce a state of profound non-responsiveness to melanoma and alloreactive T cells [58,59]. IL-10 acts directly on T cells during activation through CD28 and can be reversed with exogenous IL-2 [60]. Molecularly, IL-10 inhibits tyrosine phosphorylation of CD28 and association of PI3-kinase to CD28. These recent findings suggest that the systemic and locally elevated levels of IL-10 often found in cancer patients may further contribute to tolerance to tumor-associated antigens. Little is known of the role of IL-10 in suppressing immunity to breast cancer; however, IL-10 levels are elevated in pancreatic [61] and melanoma [62] cancer patients, and elevated levels of IL-10 correlate with poor survival [62].

Tumors can also evade immune recognition by immunologic ignorance, which reflects the inability of naïve antigen-specific T cells to recognize tumor tissue. Ochsenbein and colleagues studied immune surveillance against solid tumors and observed that transplantation of tumor pieces into mice grew readily. However, if the same tumor

cells were injected as a single-cell suspension, a protective cytolytic response was observed. It was observed that tumor growth correlated with failure of tumor cells to reach the draining lymph nodes and the absence of primed cytotoxic T cells [63]. Ignorance to antigen-bearing tumor is also observed in the OT-1 mice which are transgenic for OVA-specific T cells. Adoptive transfer of naïve OT-1 cells into OVA-expressing tumor-bearing mice does not inhibit tumor growth even though OT-1 cells are able to lyse OVA-tumors in vitro. However, if the OT-1 cells were pre-activated prior to transfer, tumor protection was observed. Additionally, mice vaccinated with OVA-pulsed APC (i.e. cross-priming) could activate naïve OT-1 cells, which could protect against tumor. These results demonstrate that ignorance plays an important role in tumor evasion and that therapeutic intervention to activate tumor-specific T cells is necessary to overcome ignorance. Thus, while some tumors may anergize T cells, others may avoid immune recognition through ignorance [64].

Tumors can induce T cell dysfunction and even death. The CD95/CD95L (Fas/FasL) system plays an important role in limiting the immune response. CD95/CD95L interactions result in apoptosis of CD95-expressing cells mediated by crosslinking of CD95 by CD95L. Muschen and colleagues studied 40 breast malignancies for the expression of CD95L [65]. They observed that CD95L expression was positively correlated with the grade of malignancy, with stage IV disease showing the highest rate of expression. In addition, in histologic analysis, they found that T cells within close proximity of tumor cells were predominantly apoptotic. The authors concluded that tumor cell-induced apoptosis of tumor-associated T cells is a potential mechanism of immunosuppression in breast cancer. To test this hypothesis, the investigators, in a separate study, examined apoptosis of Jurkat T cells following exposure to breast cancer cell lines [66]. They observed that the rate of apoptosis of Jurkat T cells was positively correlated with the levels of CD95L expressed on the tumor cell lines. Apoptotic effects were also enhanced by the addition of IFNγ suggesting a detrimental effect of this cytokine at the tumor site. Moreover, in cancer patients, depletion of $CD4^+$ and $CD8^+$ T cells in the peripheral blood was significantly correlated with CD95 expression, suggesting both systemic and local immunosuppression.

Another mechanism of tumor-associated T cell dysfunction is acquired impairment in the signaling ability of the T cells. For example, in one study 9 of 14 breast cancer patients had impaired levels of one or more signaling molecules: zeta-chain, ZAP-70, p56lck, and MAP kinase phosphatase I [67]. This generalized immunosuppression is, at least partially, mediated by the accumulation of immature myeloid cells, $Gr\text{-}1^+$ [68]. The TCR zeta-chain can be reduced in T cells by direct contact with $Gr\text{-}1^+$ myeloid cells [69]. More recently, Gabrilovich and colleagues found that $Gr\text{-}1^+$ cells purified from tumor-bearing mice directly inhibited tumor-antigen-specific CD8 T cells and had no impact on tumor-antigen-specific $CD4^+$ T cells [70]. The effects of $Gr\text{-}1^+$ cells depended on direct contact of the T cells with the $Gr\text{-}1^+$ cells and could be abolished by inclusion of APC maturation factors (e.g. GM-CSF). These results suggest that therapies designed to promote the differentiation of myeloid cells, and hence, reverse zeta chain defects may improve the success of therapies targeting activation of tumor-specific T cells.

5. Immune-based strategies in minimal disease

Vaccine strategies for breast cancer are currently being designed to overcome tolerance and generate lasting immunity. As more effective immunization techniques are developed, the clinical application of breast cancer vaccines is being focused on patients with minimal disease. Vaccines targeting breast cancer are often administered in the presence of bulky disease and have had limited therapeutic success. Extrapolating from application of active immunization in infectious diseases,

vaccines such as those targeting chicken pox or influenza are given in the absence of infection and have only limited efficacy if administered after exposure to the pathogen. The goal of vaccination against breast cancer may be to elicit significant immunologic memory capable of rapidly expanding an antigen-specific T cell population in the presence of low levels of antigen encountered during cancer onset or early relapse. Indeed, vaccination against breast cancer will likely only be effective when disease is either absent or below the limit of detection. The two primary reasons for vaccinating in the absence of bulky disease are (1) patients with existing disease are often immunosuppressed, and (2) the response to repeated vaccination in the absence of disease is limited and reaches a plateau level that is insufficient to eradicate existing malignancy before death occurs.

However, vaccination in the presence of some tumor may be beneficial during generation of the immune response due to epitope spreading, apoptosis, and necrosis of tumor cells. Epitope spreading is an amplification of the immune response that results from extending immunity from one antigen to other secondary antigens also expressed by the tumor cells. Epitope, or determinant spreading, is a phenomenon first described in autoimmune diseases including Theiler's murine encephalomyelitis virus-induced demyelinating disease, murine experimental autoimmune encephalitis, and diabetes in non-obese diabetic mice [71]. It is thought that initiating an immune response against a single antigen can elicit inflammation that leads to tissue damage. Tissue debris is taken up and other tissue antigens are cross-presented by APC to $CD4^+$ T cells in the regional lymph nodes. These newly recruited antigen-specific $CD4^+$ T cells could further exacerbate tissue destruction. In the setting of minimal residual tumor, inflammation at the tumor site induced by vaccination could potentially broaden the immune response as is seen in autoimmune disease. Although previous observations of epitope spreading have been confined to the $CD4^+$ immune effector arm, recent studies by Markiewicz and colleagues demonstrated that epitope spreading can occur following immunization with MHC class I-restricted peptides in the P815 tumor model. They observed that immunization against a single MHC class I-restricted peptide resulted in rejection of both $P1A^+$ tumors and $P1A^-$ tumors [72]. Analysis of the CTL response revealed that $P1A^+$ immunization resulted in intermolecular epitope spreading of the immune response to another P815 tumor antigen, P1E. Thus, epitope spreading is a phenomenon applicable to both the $CD4^+$ and $CD8^+$ T cells. These results suggest that broadening the immune response with epitope spreading in the presence of tumor could potentially result in the generation of immunity that may minimize the emergence of antigen-loss variants [72].

Vaccination strategies to successfully immunize against breast cancer are varied. All techniques focus on enhancing the immunogenicity of the tumor cell or of a particular breast cancer-associated antigen. Vaccines that have been translated to the clinic include intact tumor cells transfected with co-stimulatory molecules to enhance immunogenicity, glycoprotein-based vaccines given directly after the administration of drugs such as cytoxan to alter the immune microenvironment, and protein-based vaccines administered with multiple adjuvants to create local inflammatory responses to augment the function of APC [73]. Methods of immunization that directly address the inadequacies of the pre-existent immune response to breast cancer are more likely to result in successful immunization. One area of vaccine development, focused on augmenting APC function, is the use of DC as vaccine adjuvants.

Much attention has been focused on the activation of DC during vaccination since DC initiate and sustain T cell-mediated immune responses. Target antigens can be delivered directly to ex vivo cultured DC followed by subsequent injection of the DC-based vaccine. DC can also be modulated in vivo by using adjuvants that tar-

get DC such as GM-CSF. Ex vivo generation of antigen-pulsed DC has been used extensively in vaccination strategies and can result in the generation of substantial long-lived immunity [74]. For example, Brossart and colleagues conducted a small phase I/II trial to assess both the clinical and immunologic consequences of vaccinating breast and ovarian cancer patients with one of two vaccines consisting of ex vivo derived DC pulsed with either the HER-2/neu HLA-A2 peptides or MUC-1 HLA-A2 binding peptides [75]. Of 10 patients immunized, 5 developed peptide-specific responses as assessed by staining for intracellular IFNγ production by T cells. The peptide-specific T cells were also able to lyse HLA-matched HER-2/neu overexpressing tumor cell lines. Furthermore, in some of the responding patients, intermolecular epitope spreading occurred to CEA and MAGE-3, both of which are aberrantly expressed in some breast cancers, indicating potential inflammation at the tumor site as a result of vaccination. Vaccination resulted in partial regression in one patient and disease stabilization in another two patients. The disappointing clinical results are consistent with results of other trials and support the hypothesis that vaccines will likely be more efficacious in disease prevention rather than treatment of existing macroscopic disease.

Although the results of various DC immunization trials appear promising, the procedures used for isolating and loading DC are tedious and not yet broadly applicable to clinical practice [76]. The disadvantages of using DC loaded or pulsed with tumor-associated antigens include the uncertainty regarding the longevity of antigen presentation, the HLA restriction by the patient haplotype, and the relatively low number of known MHC class I, and in particular, MHC class II T helper related epitopes. Whole tumor cell preparations such as tumor lysates, apoptotic tumor cells, or DC–tumor cell fusions depend on the availability of tumor cells. In addition, the cytokines used in vitro to expand DC may affect their phagocytic activity and ability to migrate to DLN [77]. Given the problems with ex vivo generation of DC, a potential solution to the difficulties of DC culture would be to mobilize DC in vivo. Langerhans cells (LC) are skin DC and are the most effective APC present in the skin. The soluble factors present in the dermis that recruit and mature skin DC, LC are well known [78]. Similar to other APC, the LC role is to recognize, internalize, and process antigen encountered in the skin and to transport antigen to the DLN for T cell recognition. As LC move from the skin to the DLN they lose their ability to process antigen and express high levels of MHC and co-stimulatory molecules such as CD80 and CD86 [78]. The functional maturation of LC is affected by epidermal cytokines, in particular GM-CSF, IL-1β, and TNFα and by stimulation with antigen mediated, in part, by co-stimulatory molecules.

GM-CSF, administered intradermally, can act as a recruitment and maturation factor for LC [79,80]. GM-CSF, as an adjuvant can mobilize skin DC in vivo and has been used in vaccines targeting patients with breast cancer. Our laboratory has been conducting phase I clinical vaccine trials using peptide-based vaccines in breast and ovarian cancer patients with HER-2/neu-overexpressing tumors [74]. The HER-2/neu peptide-based vaccines consisted of HLA-class II binding peptides admixed with GM-CSF. Our initial clinical vaccination strategies have concentrated on eliciting a $CD4^+$ T helper response; vigorous T helper response may serve to augment the production of HER-2/neu antibodies and/or HER-2/neu-specific CTL, both of which could be therapeutic. In addition, $CD4^+$ T cells play a major role in the maintenance of immunologic memory. Patients were immunized once a month for 6 months, and 38 patients completed the planned six vaccinations [81]. The majority of patients could be immunized to at least one of the peptides in their vaccine, indicative of immune competence of the selected population. In addition, over half of the patients developed HER-2/neu protein-specific immunity after peptide immunization. The HER-2/neu protein-

specific T cell responses were similar in magnitude to responses generated to a foreign antigen, keyhole limpet hemocyanin. An interesting finding of this study was that greater than 75% of the patients who completed the course of vaccinations developed intramolecular epitope spreading to HER-2/neu peptides not contained within their vaccine preparation. Strikingly, epitope spreading was significantly associated with the development of HER-2/neu protein-specific immunity, consistent with the notion that epitope spreading is a function of natural endogenous processing of antigen [81]. In addition, immunity to HER-2/neu protein was durable and lasted for at least 1 year following the end of the vaccinations. An additional arm of the study evaluated immunization of patients with helper epitopes that encompassed within their natural sequence HLA-A2 binding motifs. The rationale for this approach is that $CD4^+$ T cell help is required for the generation of $CD8^+$ T cell responses that are long-lived. We examined immune responses to both the helper and HLA-A2 peptides in HLA-A2 patients selected to receive this vaccine formulation. The majority of patients developed both HER-2/neu peptide- and protein-specific T cell immunity. Peptide responses could be identified to both the longer class II epitopes as well as the HLA-A2 binding peptides. The peptide-specific $CD8^+$ T cells were able to lyse HLA-matched HER-2/neu expressing tumor cells, and significant precursor frequencies persisted for greater than 1 year. These results demonstrate that durable T cell immunity can be generated in breast cancer patients and that vaccines can be developed that are capable of stimulating natural processing and presentation of antigen in vivo.

6. Immune-based strategies in advanced disease

The role of breast cancer vaccines may be in preventing cancer relapse or even, eventually, the development of tumors. However, the use of cancer vaccines as a treatment modality in eradicating established malignancy is questionable. Extrapolating from infectious disease models, T cell precursor frequencies after influenza immunization may range from 1:25,000 to 1:5000. However, during an active infection, the antigen-specific T cell precursor frequency may achieve levels of 1:50 circulating T cells. Immune-based strategies in advanced stage breast cancer must focus on increasing the tumor-specific T cell precursor frequency to a therapeutic rather than protective level. Examples of therapeutic interventions that may potentially be effective in eradicating existing breast cancer include antigen-specific adoptive T cell therapy and the generation of a mixed chimera in vivo stimulating a graft vs. tumor effect via allotransplantation.

The primary purpose of adoptive T cell therapy is to augment T cell responses over and above that achievable by vaccination alone. Clearly, vaccination can increase the number of immune T cells capable of recognizing and responding to antigen. Repeated vaccination further increases the number of immune effector cells, but eventually a plateau of responsiveness is reached and repeated immunizations do not appreciably change this value [82]. Adoptive immunotherapy may allow levels of immunity to be achieved which may mediate an antitumor response. Adoptive transfer of T cells has resulted in the infused cells representing as many as 1:2 of the host's lymphocytes [82]. Experiments in a murine model of breast cancer have shown that vaccination alone, in the neu-transgenic mouse, is effective only for prevention of disease and not treatment of established malignancy [73]. Mice, vaccinated with a neu-specific peptide-based vaccine, are able to resist a tumor challenge following the course of immunization. In contrast, if vaccination is started on the same day as a tumor implant is placed, tumors grow at the same rate in vaccinated as in control mice. Therefore, in vivo expansion of antigen-specific T cells must be limited or even suppressed. One potential method of increasing the number of antigen-specific T cells to the level needed to eliminate tumor is to expand T cells

ex vivo followed by reinfusion. T cells derived from neuTg mice immunized against neu were expanded in vitro with neu peptides [73]. The neu-specific lymphocytes were then infused into tumor-bearing mice resulting in tumor regression. Splenocytes from non-immunized animals were infused as a control and had no antitumor effect. Although adoptive immunotherapy has been successful in eradicating established disease in animal models of breast cancer [73] the approach has not been successfully translated to human clinical trials. Obstacles to the development of successful T cell therapy for human breast cancer have been the lack of (1) defined tumor antigens which would allow expansion of antigen-specific T cells, (2) a detailed understanding of the in vitro expansion requirements of T cells which would allow the generation of maximal numbers while retaining optimal antigen-specific function, and (3) understanding the in vivo environment necessary for sustaining expansion in vivo. Over the last several years, several breast cancer tumor antigens such as HER-2/neu have been defined by virtue of a pre-existent immune response against that antigen in cancer patients [3,10,83]. In addition, the cytokine environment needed to expand a functional antitumor population and sustain it in vivo has been elucidated to some degree [82].

Recent studies have shown that it is technically feasible to readily expand breast antigen-specific T cells from patients who have been immunized with breast cancer vaccines [84]. The ease of ex vivo isolation and expansion may be related to a starting precursor frequency that has been boosted by vaccination [85]. As an example, a patient developed a significant increase in $CD8^+$ precursor frequency to HER-2/neu HLA-A2 binding peptide, p369–377, after active immunization. Following vaccination, T cell clones specific for p369–377 were isolated by limiting dilution and characterized. A total of 21 p369–377 clones were generated from this patient. With the exception of two clones, all clones were $CD3^+$. Eleven of the clones were $CD8^+/CD4^-$. Nine of the clones were $CD4^+/CD8^-$, despite being specific for an HLA-A2 binding peptide. The remaining five clones contained varying levels of both CD4 and CD8. The majority (19/21) of clones expressed the α/β TCR, but interestingly, two clones expressed the γ/δ TCR. Several of these clones could be induced to secrete IFNγ in response to p369–377 peptide stimulation. Several clones could lyse HLA-A2-transfected HER-2/neu-overexpressing tumor cells, including the γ/δ TCR expressing clones. A similar report by Reddish et al., demonstrates that breast cancer patients can generate MHC class I-restricted CTL against MUC-1-expressing adenocarcinomas following vaccination with a MUC-1 helper peptide [86]. Investigations such as these demonstrate that ex vivo expansion and characterization of breast cancer-specific T cells is most likely facilitated by vaccination and may lay the foundation for the use of antigen-specific T cell infusions for the treatment of advanced stage breast cancer.

Another therapeutic intervention that may greatly increase the tumor-specific T cell precursor frequency is allogeneic hematopoietic stem cell transplantation (alloHSCT) [87]. AlloHSCT has been very effective in treating hematopoietic malignancy, and a significant part of the curative potential of alloHSCT is due to reactivity of donor immune cells against host or tumor cell antigens referred to as the 'graft-versus-leukemia' or 'graft-versus-tumor' (GVT) effect [88–90]. The most compelling evidence for a cell-mediated GVT effect comes from recent observations that the infusion of allogeneic lymphocytes, or donor lymphocyte infusion (DLI), results in the remission of leukemic cells without any further cytotoxic therapy in patients who experienced relapse of disease after a traditional alloHSCT [91,92]. The effectiveness of DLI in inducing the remission of recurrent malignancy has been demonstrated for almost all hematologic malignancies although its efficacy varies across tumor subtypes [93,94].

Due to the success of allogeneic transplant in mediating an antitumor effect in hematopoi-

etic cancers, studies have been initiated investigating the approach in solid tumors such as breast cancer. Morecki and colleagues studied the effect of allogeneic adoptive T cell therapy on tumor growth in a murine transplant model using the 4T1 mammary carcinoma cell line, H-2^d [95]. Inoculation of 4T1 cells into syngeneic mice, BALB/c or (BALB/cXC57BL/6)F1, both of an H-2^d background, resulted in the development of lung tumors. Sub-lethally irradiated F1 mice were inoculated with 4T1 cells to simulate minimal residual disease. Mice then received immunocompetent splenocytes derived from naïve F1 mice, BALB/c mice that were syngeneic to the tumor but semi-allogeneic to the host, or from C57BL/6 mice that were allogeneic to the tumor and semi-allogeneic to the host. The survival of F1 tumor-bearing mice that were treated with allogeneic C57BL/6 splenocytes was significantly prolonged, as compared to mice given F1 or BALB/c-derived splenocytes syngeneic to 4T1 tumor cells. Furthermore, an efficient GVT reaction was demonstrated in vitro and in vivo with MHC-mismatched DBA/2 splenocytes from mice pre-sensitized by multiple injections of irradiated tumor or BALB/c-derived spleen cells [96].

Investigators at the National Cancer Institute used a murine metastatic breast cancer model to determine whether allo-specific donor $CD8^+$ CTL of type 2 cytokine phenotype (Tc2 cells) mediate a GVT effect with reduced GVHD, as compared to allo-specific donor $CD8^+$ CTL of type 1 cytokine phenotype (Tc1 cells) [97]. A parent-into-F1 transplant model was established using B6 H-2^b, stem cell and T cell infusions into irradiated CB6F1 hosts, H-$2^{b/d}$. Mice were inoculated with an MMTV breast cancer line, TSA H-2^d. Both Tc1 and Tc2 subsets lysed allogeneic targets, including the TSA breast cancer line. On average, transplanted mice receiving tumor but no donor T cells, died of tumor at day 29 after transplant. Transplant recipients co-injected with tumor and Tc1 cells or Tc2 cells demonstrated a statistically significant prolongation of survival relative to controls. The GVT effect, mediated by the Tc1 cells, was associated with histologic evidence of severe GVHD in all major target organs. In marked contrast, Tc2 recipients had greatly reduced histologic GVHD. These data indicate that immunocompetent cells allogeneic to the mammary carcinoma cells were able to inhibit tumor development in the primary host and to prevent tumor growth in the adoptive recipient, which suggests that allogeneic cell therapy may be an efficient antitumor therapy for breast cancer.

Although alloHSCT has shown promise as a therapeutic strategy in advanced stage renal cell carcinoma [98], whether the modality has relevant clinical implications in patients with breast cancer is still unknown but there is anecdotal clinical evidence to support this possibility [99–101]. Eibl and colleagues reported a 32-year-old woman with inflammatory breast cancer who received an allogeneic bone marrow transplant from an HLA-identical sibling [99]. The myeloablative conditioning regimen consisted of cyclophosphamide, thiotepa, and carboplatin. Resolution of the patient's liver metastases was observed simultaneously with the development of clinical GVHD in the first weeks after transplant. CTL, derived from the patient, were tested in a chromium-release assay against B and T lymphocytes of the patient and donor collected prior to transplant as well as against a panel of breast cancer cell lines. The T cells recognized host cells but not HLA-identical donor cells. Recognition was MHC class I antigen-restricted. In addition, minor histocompatibility antigen (MiHA)-specific and MHC class I antigen-restricted cytotoxic T lymphocytes recognizing breast cancer cells were isolated from the peripheral blood of the patient. Four of eight breast cancer lines were recognized by CTL; three of them shared the HLA antigens HLA-A2, B44, and DR1. Similarly, Ueno and colleagues treated 10 metastatic breast cancer patients with high-dose chemotherapy (cyclophosphamide, BCNU, and thiotepa) and alloHSCT [100]. All patients engrafted and had hematologic recovery. Shortly after transplantation, one patient achieved a complete remission, five achieved a partial remis-

sion, and four had stable disease. In two patients, metastatic liver lesions regressed with the onset of acute GHVD, suggesting a GVT effect.

These studies suggest a possible GVT effect in breast cancer; however, the results are not definitive and there is significant concern over subjecting patients to the potential risks associated with conventional allogeneic transplantation. These potential risks include GVHD and major organ dysfunction as a sequelae of myeloablative preparative regimens. The demonstration that an immune-mediated GVT effect plays a central role in the therapeutic efficacy of alloHSCT has led to the development of less intense conditioning regimens which are adequately immunosuppressive to permit the engraftment of donor hematopoietic stem cells. A variety of non-myeloablative conditioning regimens have been reported [102–107]. All regimens share the similar goal of providing sufficient immunosuppression to achieve allogeneic donor engraftment while attempting to minimize toxicity. Rizzieri and colleagues at Duke University have recently reported initial results using non-myeloablative alloHSCT in the setting of metastatic breast cancer [108]. Following a conditioning regimen of Campath 1H, fludarabine, and cyclophosphamide, five patients with metastatic breast cancer underwent alloHSCT. At a median follow-up of 90 days, three patients had been observed to have a partial response after transplantation. At the time of this report, four of the five patients had maintained a partial response or stable disease.

Several observations can be made from these early trials. The data indicate that engraftment of donor hematopoietic stem cells may occur with both fludarabine- and radiation-based regimens. However, as shown in other studies, there is great variability relative to the degree of donor engraftment, and graft rejection is a significant problem [102,109]. Although these regimens have provided many of the anticipated advantages, many of the problems that have been associated with myeloablative alloHSCT persist, e.g. GVHD, and will have to be addressed in future trials.

7. Conclusions

Breast cancer is an immunogenic tumor. Patients develop immune responses against specific proteins regulating the growth of their tumors, but the immunity that occurs endogenously is not effective for tumor eradication. Both the immune microenvironment and the tumor microenvironment influence the initiation and propagation of breast cancer-specific immunity. As the complex interplay between T cells, cytokines, APC, tumor cells, and other immune effectors is unraveled, immunotherapy targeting breast cancer can be more rationally designed. Clinically, the application of immune-based treatment to breast cancer is dictated by stage of disease. Recent clinical studies are demonstrating the generation of immune responses after active immunization. Hopefully, advances defining immunity to breast cancer at the bench will result in similar successes in the application of the immunotherapies in the clinic.

8. Abbreviations

APC	antigen presenting cell
CTL	cytotoxic T cell
DC	dendritic cell
DLN	draining lymph node
HLA	human leukocyte antigen
IFN	interferon
IL	interleukin
MHC	major histocompatibility
MUC	mucin
NK	natural killer
NKT	natural killer T cell
neuTg	neu-transgenic
TCR	T cell receptor
TNF	tumor necrosis factor

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References

1. Pardoll DM (1994) Tumour antigens. A new look for the 1990s. *Nature, 369*, 357.
2. Houghton AN (1994) Cancer antigens: immune recognition of self and altered self. *J Exp Med, 180*, 1–4.
3. Disis ML, Calenoff E, McLaughlin G et al. (1994) Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. *Cancer Res, 54*, 16–20.
4. Disis ML, Smith JW, Murphy AE, Chen W, Cheever MA (1994) In vitro generation of human cytolytic T-cells specific for peptides derived from the HER-2/neu protooncogene protein. *Cancer Res, 54*, 1071–1076.
5. Disis ML, Cheever MA (1996) Oncogenic proteins as tumor antigens. *Curr Opin Immunol, 8*, 637–642.
6. Nanda NK, Sercarz EE (1995) Induction of anti-self-immunity to cure cancer. *Cell, 82*, 13–17.
7. Bargmann CI, Hung MC, Weinberg RA (1986) The neu oncogene encodes an epidermal growth factor receptor-related protein. *Nature, 319*, 226–230.
8. Coussens L, Yang-Feng TL, Liao YC et al. (1985) Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science, 230*, 1132–1139.
9. Press MF, Cordon-Cardo C, Slamon DJ (1990) Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissues. *Oncogene, 5*, 953–962.
10. Disis ML, Pupa SM, Gralow JR, Dittadi R, Menard S, Cheever MA (1997) High-titer HER-2/neu protein-specific antibody can be detected in patients with early-stage breast cancer. *J Clin Oncol, 15*, 3363–3367.
11. Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, Eberlein TJ (1995) Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. *Proc Natl Acad Sci USA, 92*, 432–436.
12. Vonderheide RH, Hahn WC, Schultze JL, Nadler LM (1999) The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. *Immunity, 10*, 673–679.
13. Reddish M, MacLean GD, Koganty RR et al. (1998) Anti-MUC1 class I restricted CTLs in metastatic breast cancer patients immunized with a synthetic MUC1 peptide. *Int J Cancer, 76*, 817–823.
14. Palmisano GL, Pistillo MP, Capanni P et al. (2000) Investigation of HLA class I downregulation in breast cancer by RT-PCR. *Hum Immunol, 62*, 133–139.
15. Disis ML, Knutson KL, Schiffman K, Rinn K, McNeel DG (2000) Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. *Breast Cancer Res Treat, 62(3)*, 245–252.
16. Kalams SA, Walker BD (1998) The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *J Exp Med, 188*, 2199–2204.
17. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ (1992) Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci USA, 89*, 10578–10582.
18. Reilly RT, Gottlieb MB, Ercolini AM et al. (2000) HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. *Cancer Res, 60*, 3569–3576.
19. Reilly RT, Machiels JP, Emens LA et al. (2000) The collaboration of both humoral and cellular HER-2/neu-targeted immune responses is required for the complete eradication of HER-2/neu-expressing tumors. *Cancer Res, 61*, 880–883.
20. Surman DR, Dudley ME, Overwijk WW, Restifo NP (2000) Cutting edge: CD4+ T cell control of CD8+ T cell reactivity to a model tumor antigen. *J Immunol, 164*, 562–565.
21. Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D, Levitsky H (1998) The central role of CD4(+) T cells in the antitumor immune response. *J Exp Med, 188*, 2357–2368.
22. Disis ML, Grabstein KH, Sleath PR, Cheever MA (1999) Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. *Clin Cancer Res, 5*, 1289–1297.
23. Zeng G, Touloukian CE, Wang X, Restifo NP, Rosenberg SA, Wang RF (2000) Identification of CD4+ T cell epitopes from NY-ESO-1 presented by HLA-DR molecules. *J Immunol, 165*, 1153–1159.
24. Zeng G, Wang X, Robbins PF, Rosenberg SA, Wang RF (2000) CD4(+) T cell recognition of MHC class II-restricted epitopes from NY-ESO-1 presented by a prevalent HLA DP4 allele: association with NY-ESO-1 antibody production. *Proc Natl Acad Sci USA, 98*, 3964–3969.
25. Divino CM, Chen SH, Yang W, Thung S, Brower ST, Woo SL (2000) Anti-tumor immunity induced by interleukin-12 gene therapy in a metastatic model of breast cancer is mediated by natural killer cells. *Breast Cancer Res Treat, 60*, 129–134.
26. Shi F, Ljunggren H, Sarvetnick N (2001) Innate immunity and autoimmunity: from self-protection to self-destruction. *Trends Immunol, 22*, 97–101.
27. Nishimura T, Kitamura H, Iwakabe K et al. (2000) The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing

dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int Immunol, 12*, 987–994.
28. Liu YY, Han TY, Giuliano AE, Cabot MC (1999) Expression of glucosylceramide synthase, converting ceramide to glucosylceramide, confers adriamycin resistance in human breast cancer cells. *J Biol Chem, 274*, 1140–1146.
29. Carr A, Mullet A, Mazorra Z et al. (2000) A mouse IgG1 monoclonal antibody specific for N-glycolyl GM3 ganglioside recognized breast and melanoma tumors. *Hybridoma, 19*, 241–247.
30. Dennis JW, Granovsky M, Warren CE (1999) Glycoprotein glycosylation and cancer progression. *Biochim Biophys Acta. 1473*, 21–34.
31. Heath WR, Carbone FR (2001) Cross-presentation, dendritic cells, tolerance and immunity. *Annu Rev Immunol, 19*, 47–64.
32. Wolfers J, Lozier A, Raposo G et al. (2001) Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med, 7*, 297–303.
33. Bellone M (2000) Apoptosis, cross-presentation, and the fate of the antigen specific immune response. *Apoptosis, 5*, 307–314.
34. Bell D, Chomarat P, Broyles D et al. (1999) In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. *J Exp Med, 190*, 1417–1426.
35. Candido KA, Shimizu K, McLaughlin JC et al. (2001) Local administration of dendritic cells inhibits established breast tumor growth: implications for apoptosis-inducing agents. *Cancer Res, 61*, 228–236.
36. Chen LK, Tourvieille B, Burns GF et al. (1986) Interferon: a cytotoxic T lymphocyte differentiation signal. *Eur J Immunol, 16*, 767–770.
37. Djeu JY, Stocks N, Zoon K, Stanton GJ, Timonen T, Herberman RB (1982) Positive self regulation of cytotoxicity in human natural killer cells by production of interferon upon exposure to influenza and herpes viruses. *J Exp Med, 156*, 1222–1234.
38. Collart MA, Belin D, Vassalli JD, de Kossodo S, Vassalli P (1986) Gamma interferon enhances macrophage transcription of the tumor necrosis factor/cachectin, interleukin 1, and urokinase genes, which are controlled by short-lived repressors. *J Exp Med, 164*, 2113–2118.
39. Buchmeier NA, Schreiber RD (1985) Requirement of endogenous interferon-gamma production for resolution of Listeria monocytogenes infection. *Proc Natl Acad Sci USA, 82*, 7404–7408.
40. Nakajima C, Uekusa Y, Iwasaki M et al. (2001) A role of interferon-gamma (IFN-gamma) in tumor immunity: T cells with the capacity to reject tumor cells are generated but fail to migrate to tumor sites in IFN-gamma-deficient mice. *Cancer Res, 61*, 3399–3405.
41. Yao L, Pike SE, Setsuda J et al. (2000) Effective targeting of tumor vasculature by the angiogenesis inhibitors vasostatin and interleukin-12. *Blood, 96*, 1900–1905.
42. Braun SE, Chen K, Foster RG et al. (2000) The CC chemokine CK beta-11/MIP-3 beta/ELC/Exodus 3 mediates tumor rejection of murine breast cancer cells through NK cells. *J Immunol, 164*, 4025–4031.
43. Kim CH, Pelus LM, White JR, Applebaum E, Johanson K, Broxmeyer HE (1998) CK beta-11/macrophage inflammatory protein-3 beta/EBI1-ligand chemokine is an efficacious chemoattractant for T and B cells. *J Immunol, 160*, 2418–2424.
44. Dieu MC, Vanbervliet B, Vicari A et al. (1998) Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med, 188*, 373–386.
45. Kim CH, Pelus LM, Appelbaum E, Johanson K, Anzai N, Broxmeyer HE (1999) CCR7 ligands, SLC/6Ckine/Exodus2/TCA4 and CKbeta-11/MIP-3beta/ELC, are chemoattractants for CD56(+)-CD16(−) NK cells and late stage lymphoid progenitors. *Cell Immunol, 193*, 226–235.
46. Esche C, Cai Q, Peron JM et al. (2000) Interleukin-12 and Flt3 ligand differentially promote dendropoiesis in vivo. *Eur J Immunol, 30*, 2565–2575.
47. Josien R, Li HL, Ingulli E et al. (2000) TRANCE, a tumor necrosis factor family member, enhances the longevity and adjuvant properties of dendritic cells in vivo. *J Exp Med, 191*, 495–502.
48. Fiebiger E, Meraner P, Weber E et al. (2001) Cytokines regulate proteolysis in major histocompatibility complex class II-dependent antigen presentation by dendritic cells. *J Exp Med, 193*, 881–892.
49. Lynch DH, Andreasen A, Maraskovsky E, Whitmore J, Miller RE, Schuh JC (1997) Flt3 ligand induces tumor regression and antitumor immune responses in vivo. *Nat Med, 3*, 625–631.
50. Brunda MJ, Luistro L, Warrier RR et al. (1993) Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J Exp Med, 178*, 1223–1230.
51. Lah TT, Hawley M, Rock KL, Goldberg AL (1995) Gamma-interferon causes a selective induction of the lysosomal proteases, cathepsins B and L, in macrophages. *FEBS Lett, 363*, 85–89.
52. Coulie PG, Ikeda H, Baurain JF, Chiari R (1999) Antitumor immunity at work in a melanoma patient. *Adv Cancer Res, 76*, 213–242.
53. Sprent J, Kishimoto H (2001) The thymus and central tolerance. *Philos Trans R Soc Lond B Biol Sci, 356*, 609–616.

54. Lechler R, Chai JG, Marelli-Berg F, Lombardi G (2001) T-cell anergy and peripheral T-cell tolerance. *Philos Trans R Soc Lond B Biol Sci, 356*, 625–637.
55. Wolfram RM, Budinsky AC, Brodowicz T et al. (2000) Defective antigen presentation resulting from impaired expression of costimulatory molecules in breast cancer. *Int J Cancer, 88*, 239–244.
56. Meyer GC, Batrla R, Rudy W et al. (1999) Potential of CD80-transfected human breast carcinoma cells to induce peptide-specific T lymphocytes in an allogeneic human histocompatibility leukocyte antigens (HLA)-A2.1+-matched situation. *Cancer Gene Ther, 6*, 282–288.
57. Ohlen C, Kalos M, Hong DJ, Shur AC, Greenberg PD (2001) Expression of a tolerizing tumor antigen in peripheral tissue does not preclude recovery of high-affinity CD8+ T cells or CTL immunotherapy of tumors expressing the antigen. *J Immunol, 166*, 2863–2870.
58. Becker JC, Czerny C, Brocker EB (1994) Maintenance of clonal anergy by endogenously produced IL-10. *Int Immunol, 6*, 1605–1612.
59. Groux H, Bigler M, de Vries JE, Roncarolo MG (1996) Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J Exp Med, 184*, 19–29.
60. Joss A, Akdis M, Faith A, Blaser K, Akdis CA (2000) IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway. *Eur J Immunol, 30*, 1683–1690.
61. von Bernstorff W, Voss M, Freichel S et al. (2001) Systemic and local immunosuppression in pancreatic cancer patients. *Clin Cancer Res, 7*, 925s–932s.
62. Nemunaitis J, Fong T, Shabe P, Martineau D, Ando D (2001) Comparison of serum interleukin-10 (IL-10) levels between normal volunteers and patients with advanced melanoma. *Cancer Invest, 19*, 239–247.
63. Ochsenbein AF, Klenerman P, Karrer U et al. (1999) Immune surveillance against a solid tumor fails because of immunological ignorance. *Proc Natl Acad Sci USA, 96*, 2233–2238.
64. Dalyot-Herman N, Bathe OF, Malek TR (2000) Reversal of CD8+ T cell ignorance and induction of anti-tumor immunity by peptide-pulsed APC. *J Immunol, 165*, 6731–6737.
65. Muschen M, Moers C, Warskulat U et al. (1999) CD95 ligand expression in dedifferentiated breast cancer. *J Pathol, 189*, 378–386.
66. Uschen M, Moers C, Warskulat U, Even J, Niederacher D, Beckmann MW (2000) CD95 ligand expression as a mechanism of immune escape in breast cancer. *Immunology, 99*, 69–77.
67. Kurt RA, Urba WJ, Smith JW, Schoof DD (1998) Peripheral T lymphocytes from women with breast cancer exhibit abnormal protein expression of several signaling molecules. *Int J Cancer, 78*, 16–20.
68. Almand B, Clark JI, Nikitina E et al. (2001) Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol, 166*, 678–689.
69. Otsuji M, Kimura Y, Aoe T, Okamoto Y, Saito T (1996) Oxidative stress by tumor-derived macrophages suppresses the expression of CD3 zeta chain of T-cell receptor complex and antigen-specific T-cell responses. *Proc Natl Acad Sci USA, 93*, 13119–13124.
70. Gabrilovich DI, Velders MP, Sotomayor EM, Kast WM (2001) Mechanism of immune dysfunction in cancer mediated by immature gr-1(+) myeloid cells. *J Immunol, 166*, 5398–5406.
71. Vanderlugt CJ, Miller SD (1996) Epitope spreading. *Curr Opin Immunol, 8*, 831–836.
72. Markiewicz MA, Fallarino F, Ashikari A, Gajewski TF (2001) Epitope spreading upon P815 tumor rejection triggered by vaccination with the single class I MHC-restricted peptide P1A. *Int Immunol, 13*, 625–632.
73. Knutson KL, Disis ML (2001) Expansion of HER-2/neu specific T cells ex vivo following immunization with a HER-2/neu peptide-based vaccine. *Clin Breast Cancer, 2*, 73–79.
74. Weber JS, Mule JJ (2001) How much help does a vaccine-induced T-cell response need? *J Clin Invest, 107*, 553–554.
75. Brossart P, Wirths S, Stuhler G, Reichardt VL, Kanz L, Brugger W (2000) Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. *Blood, 96*, 3102–3108.
76. Fong L, Engleman EG (2000) Dendritic cells in cancer immunotherapy. *Annu Rev Immunol, 18*, 245–273.
77. Banchereau J, Briere F, Caux C et al. (2000) Immunobiology of dendritic cells. *Annu Rev Immunol, 18*, 767–811.
78. Kimber I, Cumberbatch M, Dearman RJ, Bhushan M, Griffiths CE (2000) Cytokines and chemokines in the initiation and regulation of epidermal Langerhans cell mobilization. *Br J Dermatol, 142*, 401–412.
79. Kaplan G, Walsh G, Guido LS et al. (1992) Novel responses of human skin to intradermal recombinant granulocyte/macrophage-colony-stimulating factor: Langerhans cell recruitment, keratinocyte growth, and enhanced wound healing. *J Exp Med. 175*, 1717–1728.
80. Disis ML, Bernhard H, Shiota FM et al. (1996) Granulocyte-macrophage colony-stimulating factor: an effective adjuvant for protein and peptide-based vaccines. *Blood, 88*, 202–210.
81. Disis ML, Gooley TA, Rinn K, Davis D, Piepkorn M, Cheever MA, Knutson KL, Schiffman K (2002) Intramolecular epitope spreading to the HER-2/neu

protein occurs after active immunization with HER-2/neu peptide-based vaccine. (In preparation).
82. Cheever MA, Chen W (1997) Therapy with cultured T cells: principles revisited. *Immunol Rev, 157*, 177–194.
83. Disis ML, Cheever MA (1998) HER-2/neu oncogenic protein: issues in vaccine development. *Crit Rev Immunol, 18*, 37–45.
84. Knutson KL, Crosby P, Disis ML (2000) Isolation of HER-2/neu (HER2) specific T cell clones from a cancer patient following immunization with a HER2 peptide vaccine. *Proc Am Assoc Cancer Res, 40*, 310a.
85. Knutson KL, Schiffman K, Disis ML (2001) Immunization with a HER-2/neu helper peptide vaccine generates of HER-2/neu CD8 T cell immunity in cancer patients. *J Clin Invest, 107*, 477–484.
86. Reddish M, MacLean GD, Koganty RR et al. (1998) Anti-MUC1 class I restricted CTLs in metastatic breast cancer patients immunized with a synthetic MUC1 peptide. *Int J Cancer, 76*, 817–823.
87. Armitage JO (1994) Bone marrow transplantation. *N Engl J Med, 330*, 827–838.
88. Buckner CD, Clift RA, Fefer A et al. (1974) Marrow transplantation for the treatment of acute leukemia using HL-A-identical siblings. *Transplant Proc, 6*, 365–366.
89. Horowitz MM, Gale RP, Sondel PM et al. (1990) Graft-versus-leukemia reactions after bone marrow transplantation. *Blood, 75*, 555–562.
90. Maraninchi D, Gluckman E, Blaise D et al. (1987) Impact of T-cell depletion on outcome of allogeneic bone-marrow transplantation for standard-risk leukaemias. *Lancet*, 2, 175–178.
91. Porter DL, Roth MS, McGarigle C, Ferrara JL, Antin JH (1994) Induction of graft-versus-host disease as immunotherapy for relapsed chronic myeloid leukemia. *N Engl J Med, 330*, 100–106.
92. Mackinnon S, Papadopoulos EB, Carabasi MH et al. (1995) Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood, 86*, 1261–1268.
93. Kolb HJ, Schattenberg A, Goldman JM et al. (1995) Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients: European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia. *Blood, 86*, 2041–2050.
94. Collins RHJ, Shpilberg O, Drobyski WR et al. (1997) Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. *J Clin Oncol, 15*, 433–444.
95. Morecki S, Yacovlev E, Diab A, Slavin S (1998) Allogeneic cell therapy for a murine mammary carcinoma. *Cancer Res, 58*, 3891–3895.
96. Morecki S, Yacovlev E, Gelfand Y, Uzi I, Slavin S (2001) Cell therapy with preimmunized effector cells mismatched for minor histocompatible antigens in the treatment of a murine mammary carcinoma. *J Immunother, 24*, 114–121.
97. Fuzak J, Jung U, Foley J, Eckhaus MA, Fowler DH (2000) Allospecific Tc2 cells mediate a graft-versus-tumor effect without ongoing GVHD in a murine model of metastatic breast cancer. *Blood, 96*, 551a.
98. Childs R, Chernoff A, Contentin N et al. (2000) Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. *N Engl J Med, 343*, 750–758.
99. Eibl B, Schwaighofer H, Nachbaur D et al. (1996) Evidence for a graft-versus-tumor effect in a patient treated with marrow ablative chemotherapy and allogeneic bone marrow transplantation for breast cancer. *Blood, 88*, 1501–1508.
100. Ueno NT, Rondon G, Mirza NQ et al. (1998) Allogeneic peripheral-blood progenitor-cell transplantation for poor-risk patients with metastatic breast cancer. *J Clin Oncol, 16*, 986–993.
101. Or R, Ackerstein A, Nagler A et al. (1998) Allogeneic cell-mediated immunotherapy for breast cancer after autologous stem cell transplantation: a clinical pilot study. *Cytokines Cell Mol Ther, 4*, 1–6.
102. Giralt S, Estey E, Albitar M et al. (1997) Engraftment of allogeneic hematopoietic progenitor cells with purine analog-containing chemotherapy: harnessing graft-versus-leukemia without myeloablative therapy. *Blood, 89*, 4531–4536.
103. Slavin S, Nagler A, Naparstek E et al. (1998) Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood, 91*, 756–763.
104. Khouri IF, Keating M, Korbling M et al. (1998) Transplant-lite: induction of graft-versus-malignancy using fludarabine-based nonablative chemotherapy and allogeneic blood progenitor-cell transplantation as treatment for lymphoid malignancies. *J Clin Oncol, 16*, 2817–2824.
105. Childs R, Clave E, Contentin N et al. (1999) Engraftment kinetics after nonmyeloablative allogeneic peripheral blood stem cell transplantation: full donor T-cell chimerism precedes alloimmune responses. *Blood, 94*, 3234–3241.
106. Spitzer TR, McAfee S, Sackstein R et al. (2000) Intentional induction of mixed chimerism and achievement of antitumor responses after nonmyeloablative conditioning therapy and HLA-matched donor bone marrow transplantation for refractory hematologic malignancies. *Biol Blood Marrow Transplant, 6*, 309–320.

107. Sandmaier BM, McSweeney P, Yu C, Storb R (2000) Nonmyeloablative transplants: preclinical and clinical results. *Semin Oncol*, 27, 78–81.
108. Rizzieri DA, Long GD, Vredenburgh JJ et al. (2000) Chimerism mediated immunotherapy using CAMPATH T cell depleted peripheral blood progenitor cells (PBPC) with nonablative therapy provides reliable, durable allogeneic engraphment. *Blood*, 96, 521a.
109. McSweeney PA, Niederwieser D, Shizuru JA et al. (2001) Hematopoietic cell transplantation in older patients with hematologic malignancies: replacing high-dose cytotoxic therapy with graft-versus-tumor effects. *Blood*, 97, 3390–3400.

Neu antigen-negative variants can be generated following neu-specific antibody therapy in neu-transgenic mice[1]

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ABSTRACT

Prolonged administration of HER-2/neu-specific monoclonal antibody therapy is now widely used for the treatment of HER-2/neu-overexpressing tumors in advanced-stage breast cancer patients. Monoclonal antibody therapy has the potential to promote reduced tumor expression of HER-2/neu by receptor down-modulation and/or the generation of antigen-negative variants. Loss of antigen by either mechanism could potentially impact subsequent therapeutic strategies targeting HER-2/neu In this study the effects of chronic neu-specific monoclonal antibody therapy on tumor growth and neu protein expression were examined in a murine model of neu-overexpressing breast cancer. Treatment of neu-overexpressing tumors with neu-specific antibody, *in vitro* or *in vivo*, resulted in significant tumor growth inhibition. When neu antibody was used to treat neu-overexpressing tumor cells both *in vitro* and *in vivo* in tumor-bearing mice, neu receptor expression was not diminished after cessation of therapy. However, in the setting of clinically undetectable disease in a minority of animals, antigen-negative variants were generated. An understanding of the effects of monoclonal antibodies on target antigen expression is critical for the future design and testing of novel HER-2/neu targeted therapies administered in combination with or following HER-2/neu-specific monoclonal antibody therapy.

INTRODUCTION

HER-2/neu is an oncoprotein that is commonly overexpressed on a variety of different types of tumors. Approximately 20-30% of human breast cancers overexpress HER-2/neu. Patients with HER-2/neu-overexpressing breast cancer have poorer overall survival rates and shorter times to disease progression than compared to patients whose tumors do not overexpress HER-2/neu (1). HER-2/neu is also associated with increased resistance to chemotherapy (2). Because of these clinical characteristics of HER-2/neu protein expression in tumors, great effort is being applied to developing therapies that specifically target HER-2/neu.

HER-2/neu-specific monoclonal antibody (i.e. trastuzumab) therapy has recently been approved for clinical use and is now a standard of care for the treatment of advanced breast cancers that overexpress the HER-2/neu oncoprotein (3). Other emerging HER-2/neu-targeted therapies therefore will most likely be tested in combination with anti-HER-2/neu monoclonal antibody therapy. In general, monoclonal antibody therapy can the alter the cell surface expression of the target antigen by one of two major mechanisms. The first mechanism is receptor down-modulation, a process by which antibody induces internalization and degradation resulting in reduced or nonexistent expression of the receptor at the cell surface. This mechanism is rapid, occurring within minutes, and is reversible upon removal of antibody (4). The second mechanism is antigen-negative variant selection which results in the outgrowth of a cell population that permanently expresses little or no receptor. The development of neu antigen-negative variants after successful monoclonal antibody therapy would have a great impact on the clinical efficacy of subsequent neu-directed therapies.

In this study, we evaluated the effects of multiple doses of neu-specific antibody on tumor growth and assessed the impact of the therapy on neu protein expression (5). The growth inhibitory effects of anti-neu antibody, 7.16.4, were evaluated in a transgenic (neu-tg) mouse model of neu-overexpressing breast cancer in various disease settings from minimal disease to advanced established malignancy.

MATERIALS AND METHODS

Animals. A colony of neu-transgenic (neu-tg)[3] mice [strain name: FVB/N-TgN (MMTVneu)202Mul] was established from breeder pairs obtained from Charles River Laboratory (Bar Harbor, ME) (5). The mice harbor the non-mutated, non-activated rat neu proto-oncogene under control of the MMTV promoter. Only female mice, 8-12 weeks old, were used for experimentation. Animal care and use was in accordance with the University of Washington institutional guidelines.

Reagents. Fetal calf serum (FCS) was obtained from Gemini Bioproducts (Woodland, CA). RPMI-1640, PBS, penicillin-streptomycin, and L-glutamine were obtained from Gibco-BRL (Grand Island, NY). Monoclonal antibody 7.16.4, a mouse IgG2a antibody reactive with the rat neu oncogene-encoded p185 molecule was generously provided by Dr. Mark Greene and has been previously described (6). Rat anti-mouse IgG-FITC antibody was obtained from Pharmingen (San Diego, CA) and 4G10 antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit anti-neu antibody (Ab-1) was obtained from Oncogene Research Products (Cambridge, MA). Enhanced chemiluminescence reagents and ECL film were from Amersham International (Oakville, Ontario). Ethylenediaminetetraacetate acid (EDTA) was obtained from Ambion (Austin, TX). The protein quantification kit, Protein_{DC}, was obtained from BioRad (Hercules, CA).

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[3] The abbreviations used are: monoclonal antibody, monoclonal antibody; neu-tg, neu-transgenic; MMC, murine mammary carcinoma; ANV, antigen negative variant.

Cell lines. Mouse mammary carcinoma (MMC) cell line was established from a spontaneous tumor harvested from the neu-tg mice. MMC cells were grown and maintained in RPMI supplemented with 20% FCS as well as penicillin/streptomycin and L-glutamine. Antigen negative variant (ANV) is a cell line derived from a neu-loss variant tumor and is maintained in culture identical to MMC.

Tumor growth *in vitro* and *in vivo*. For *in vitro* experiments 1.0 x 10^5 MMC or ANV were plated in 6 well plates with media alone, mouse IgG2a (Sigma Aldrich, St. Louis, MO), or various doses of 7.16.4. Cells were harvested with a NaCl solution (0.8%) with 2 mM EDTA, washed, counted, and prepared for flow cytometry or western blot analysis. For *in vivo* tumor growth, MMC cells were harvested using 2 mM EDTA and washed prior to injection. Eight to twelve week old female neu-tg mice were inoculated with 6 x 10^6 MMC cells subcutaneously on the right mid-dorsum with a 23-gauge needle, which is a dose of tumor cells that consistently results in the development of tumors in 100% of neu-tg mice. Tumors were measured every other day with vernier calipers and tumor size was calculated as the product of length x width. Data are presented as mean +/- SEM. Significance ($p< 0.05$) was determined using Student's *t* test by comparing the means of different treatment groups. Mice were treated with every other day tail vein dosing of either 100 µl PBS as control or 30 µg 7.16.4 in PBS. Tumor measurements were taken every 2-3 days during tumor growth studies.

Flow cytometry. Cultured tumor cells were removed from plates using PBS with 2 mM EDTA. Tumor cells grown *in vivo* were harvested from mice by careful dissection under sterile conditions then passed through a fine wire mesh and washed. Both *in vitro* and *in vivo* harvested cells were washed in PBS containing 1% FCS prior to labeling. Cells (0.5 to 1.0 x 10^6) were

incubated with 2-5 μl of primary antibody (control IgG2a or anti-neu 7.16.4) were added at 4°C for 30 minutes and washed 3 times followed by secondary labeling with fluorescein isothiocyanate (FITC) conjugated goat anti-rat antibody at 4°C for 30 minutes followed by 3 washes. Samples were run on a FACS Scan II and analyzed using Cell Quest software (Becton Dickinson, San Diego, CA).

Western blotting and immunoprecipitation. Whole cell lysates, prepared in lysis buffer (20 mM Tris, pH 8.0, 1% Triton X-100, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF) as previously described (7), were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine monoclonal antibody (4G10) and anti-neu (Ab-1) by previously described methods (8). Protein concentrations were determined using Bio-Rad $Protein_{DC}$ and bovine serum albumin as standard. Rat neu was immunoprecipitated from tumor cells by incubating 0.5-1.5 mg of lysate with anti-neu 7.16.4 for 2 hours at 4° followed by the addition of 50 μl of protein G-sepharose for an additional 2 hours. To assess the amount of neu protein, membranes were probed with 5 μg/ml anti-neu polyclonal antibody, Ab-1 and (1:2000 dilution) anti-mouse HRP and developed by enhanced chemiluminescence as described (8).

RESULTS

Neu-specific monoclonal antibody treatment inhibits neu-mediated tumor growth *in vitro* without loss of cell surface neu expression. Neu-specific monoclonal antibody, 7.16.4, was evaluated at varying concentrations (0, 1, 10, 50, and 100 μg/ml) for growth inhibition, *in vitro,* against neu^+ MMC and neu^- ANV as shown in Figure 1. Antibody was added only once to culture on day 0 and cell numbers were evaluated 5 days later. 7.16.4 specifically inhibited the growth of MMC but not ANV (Fig. 1). The response of MMC was dose dependent and growth at 100 μg/ml 7.16.4 antibody resulted in a cell yield of 15 ± 4 % (mean ± SD) of control cells that had been incubated in the absence of 7.16.4. In contrast, ANV were unaffected by inclusion of 7.16.4 and at 100 μg/ml the cell yield of ANV was 111 ± 2% of control ANV cells. MMC grown in the presence of 50 μg/ml isotype matched, non-specific mouse IgG resulted in only a minor incremental decrease in cell yield (87 ± 7% vs. control, data not depicted in graph).

The cell surface expression of neu on MMC was examined in cells that were treated with 7.16.4 or isotype-matched IgG *in vitro*. As shown in Figure 2A, compared with either control media (PBS) or non-specific IgG, the addition of 7.16.4 antibody did not result in changes in the mean levels of neu expression at the cell surface of MMC over the range of doses examined in Figure 1. Furthermore, changes in the total cellular levels of neu expression were not altered as assessed by immunoblotting of cellular lysates (data not shown). Representative histograms are shown in Figures 2B-2E. In Figures 2D-E, staining of 7.16.4-treated MMC with 7.16.4 and FITC-conjugated anti-mouse IgG antibody revealed no significant down-modulation of cell surface neu. FITC-α-IgG, when used alone, also stained the cells (open histograms) reflecting

the presence of the initial dose of 7.16.4. Untreated control cells (Fig. 2B) did not demonstrate binding of FITC-α-IgG. The effects of non-specific IgG treatment of MMC are shown in Figure 2C.

Neu-specific monoclonal antibody inhibits neu-mediated tumor growth *in vivo* in an established tumor setting without neu receptor down-modulation. The effect of 7.16.4 treatment on *in vivo* growth of tumors was examined as shown in Figure 3. Continuous treatment every other day with 7.16.4, starting on either day 10 or day 14 after tumor implant did not result in complete inhibition or regression but did result in a reduced rate of tumor growth in comparison to PBS control. The earlier therapy was started, the greater the impact on overall tumor growth. For example, at 20-24 days following tumor challenge, tumors of mice that were treated beginning on day 10 following challenge were approximately 90% smaller than controls while sizes of tumors in mice treated beginning on day 14 were 60% smaller. At day 24, the mean tumor sizes differed significantly from each other, control vs. day 10, $p=0.01$; control vs. day 14, $p=0.004$; day 14 vs. day 10; $p=0.01$. The cell surface expression of neu was also examined in tumors harvested from antibody treated animals (Fig. 4). There was no measurable difference in the relative mean fluorescent intensity (rMFI) of cell surface neu expression in antibody-treated animals as compared to PBS-treated animals (Fig 4A). Representative histograms are shown in Figures 4B-E. Figures 4B-D show that antibody treated tumors still maintain high levels of neu expression. The levels of neu expression in tumors from treated animals were comparable to tumors from untreated animals.

Neu antigen-negative variants can develop in some animals after treatment with a neu-specific monoclonal antibody. When therapy with 7.16.4. monoclonal antibody is initiated at the time of tumor injection tumor growth is either completely inhibited or significantly delayed (Fig. 5). The antibody-treated group received antibody injections every other day for a total of 30 days. While all of the mice that received control infusions of PBS developed tumor, none of the mice that were treated with 7.16.4 developed measurable tumors during the course of therapy (Fig. 5A). The treated mice were followed for an additional 45 (75 days total) days to evaluate potential recurrence for relapse as shown in Fig. 5B. Two of 6 (33%) mice eventually relapsed with small slow growing tumors at the tumor injection site after day 51. The remaining mice did not develop tumors up to day 75. The tumors were examined in the relapsing mice for cell surface expression of neu (Fig. 6). Compared to MMC tumor cells shown in Fig. 6A, relapse tumors (Figs. 6D-E) demonstrated no neu expression and were comparable to the ANV cell line (Fig. 6C).

DISCUSSION

In this study, we investigated the impact of chronic neu-specific monoclonal antibody therapy on the expression of the target antigen, neu. Chronic monoclonal antibody therapy can potentially induce the loss of target antigen expression at the cell surface by two major mechanisms. First, binding of antibody to cell surface molecules can result in activation-induced internalization and degradation. Second, theoretically, antigen-negative variants can arise following destruction of antigen-positive tumor cells by antibody-induced mechanims.

Antibody-induced internalization of the target antigen has been reported for a number of cell surface receptors and is a relatively rapid event occurring immediately after antibody exposure (9). Vesicles internalizing the receptors fuse with lysosomes which results in increased degradation and reduced steady state levels of the receptor (9). While not all monoclonal antibodies induce internalization, prior *in vitro* studies have demonstrated that 7.16.4 results in HER-2/neu down-modulation of neu-transformed NIH 3T3 cells (4, 10, 11). The loss of HER-2/neu expression by down-modulation significantly inhibits growth and causes reversion of the transformed phenotype (4). Receptor internalization induced by 7.16.4 in the NIH 3T3 cells is a rapid event occurring in the first hour of antibody exposure and is sustained as long as antibody is delivered (4). These findings have led to the conclusion that a major mechanism of the growth-inhibitory action of 7.16.4 is through antibody-induced internalization. *In vitro* studies with trastuzumab (Herceptin) have also led to similar conclusions (11). In the current study, in an established disease model, neu surface expression was retained. A potential explanation for why we did not observe receptor down-modulation in our studies is that receptor internalization is rapidly reversed with cessation of antibody therapy. Down-modulation of the receptor may

have been reversed during the time necessary for harvesting and analysis of the tumor cells. Another possible mechanism is that inhibition of growth signaling by receptor internalization and degradation by monoclonal antibody therapy could have induced increased receptor synthesis resulting in no detectable change in cell surface neu expression. Indeed, previous studies have shown an inverse correlation between HER-2/neu signaling and HER-2/neu protein expression. For example, Antoniotti and colleagues observed that epidermal growth factor-induced down-modulation of HER-2/neu in breast cancer cell lines is accompanied by a compensatory increase in the transcription of the HER-2/neu gene (12). Thus, in the present study, blockade of growth signals by neu with neu-specific monoclonal antibodies may have led to a compensatory increase in neu transcription that could have offset any down-modulation. In recent studies it has been demonstrated that HER-2/neu receptors can be endocytosis impaired compared to EGFR when exposed to receptor-specific monoclonal antibodies (13). It is possible that neu+ tumor cells arising in the neu-tg mouse are endocytosis defective. Recently, loss of antigen expression has been observed in stage II and III breast cancer patients undergoing preoperative trastuzumab therapy. In that study patients with HER-2/neu-overexpressing tumors were treated with trastuzumab and paclitaxel prior to surgery and adjuvant therapy (14). HER-2/neu status was determined prior to any treatment and at the time of surgery (i.e. following trastuzumab/paclitaxel therapy). Seventy-five percent of the patients had objective clinical responses to preoperative trastuzumab and paclitaxel therapy. Twenty-seven (68%) of the patients had assessable tumors following preoperative therapy, in which it was observed that 7 had significantly reduced levels of HER-2/neu staining. While the authors suggest that down-modulation may have occurred, the analyses did not appear to be extensive enough to rule out the possibility that antigen-negative variants were observed.

The generation of antigen-negative variants results from immunoselection is a significant clinical issue in the development of immune-based therapies targeting a single antigen and has been best described for clinical studies focusing on T cell-directed immunotherapy. For example, in a study by Jager and colleagues, melanoma patients treated with a MART-1 and tyrosinase peptide-based vaccine showed gradual losses in expression of both MART-1 and tyrosinase protein suggesting immunoselection (15). Only recently has there been some indication that monoclonal antibody therapy might result in the generation of antigen-negative variants. In lymphoma patients treated with monoclonal anti-CD20 antibody (i.e. rituximab) it was observed that treatment resulted in reduced CD20 expression in the tumor cells localized to the bone marrow but not those localized to lymph nodes (16). In the present study, in the established disease model, we did not see the generation of antigen-negative variants most likely because therapy was only cytostatic rather than cytolytic and the neu-positive tumors continued to grow. However, when the tumor burden was minimal, tumor growth was significantly delayed or inhibited and the animals were apparently cured for an extended period of time following discontinuation of monoclonal antibody therapy. A fraction of the animals however developed antigen-negative tumors after a long latency. The fact that these animals had been off of therapy for 20 days prior to the development of tumors argues that these tumors were true immunoselected antigen-negative variants rather than tumors with transient down-modulation. Immunoselection of antigen-negative variants represents successful immune therapy targeting a single antigen and argues that monoclonal antibody therapy should be combined with strategies targeting other antigens to minimize the risk of antigen-negative variants. Furthermore, identifying whether antigen-negative variants arise after therapy would be critical for design of future therapies for the patient that may target the same antigen, because of the efficacy of the

subsequent targeted approaches can only be interpreted in light of the tumor antigen levels present at the start of therapy.

Many strategies are being tested that specifically target HER-2/neu, including monoclonal antibody therapy, gene therapy, vaccines, tyrosine kinase inhibition, adoptive T·cell therapy, and antisense therapy (17). HER-2/neu-specific monoclonal antibody therapy (i.e. trastuzumab) has shown clinical efficacy and is now a standard of care for the treatment of HER-2/neu-overexpressing breast cancer (3). Current routine clinical use of anti-HER-2/neu monoclonal antibody therapy, for extended periods of time in advanced cancer patients, mandates that newer HER-2/neu targeting therapeutics be tested in combination with trastuzumab. The mechanism of loss of antigen expression is important. If tumors lost surface antigen expression due mainly to receptor down-modulation, monoclonal antibody therapy targeting HER-2/neu may improve other approaches such as HER-2/neu-specific adoptive T cell therapy. In an *in vitro* study, zum Buschenfelde and colleagues observed that trastuzumab pretreatment enhanced the cytolytic activity of HER-2/neu-specific T cells against HER-2/neu-overexpressing tumors (18). Results of that study suggested that it is possible that trastuzumab promotes the internalization and degradation of HER-2/neu resulting in increased presentation of HER-2/neu MHC class I epitopes which may lead to greater activation and expansion of HER-2/neu-specific T cells. A mechanism of loss of receptor expression such as the generation of antigen negative variants would render subsequent neu-directed therapies useless.

In summary, we show here a murine model of effective antibody-based therapy that does not result in neu receptor down-modulation during chronic cytostatic treatment of established disease. However, tumors that arise after chronic antibody therapy in the setting of minimal

disease are true antigen-negative variants. Thus, the development of permanent antigen-negative variants may be influenced by the disease burden present at the start of therapy. Lastly, an understanding of the effects of monoclonal antibody therapy on target antigen expression is critical for the future design and testing of novel HER-2/neu-directed therapies administered in patients undergoing HER-2/neu-specific monoclonal antibody therapy.

REFERENCES

1. Masood, S. and Bui, M. M. Prognostic and predictive value of HER2/neu oncogene in breast cancer, Microsc Res Tech. *59:* 102-8., 2002.

2. Yu, D. Mechanisms of ErbB2-mediated paclitaxel resistance and trastuzumab- mediated paclitaxel sensitization in ErbB2-overexpressing breast cancers, Semin Oncol. *28:* 12-7., 2001.

3. Baselga, J. Clinical trials of Herceptin(R) (trastuzumab), Eur J Cancer. *37 Suppl 1:* 18-24., 2001.

4. Drebin, J. A., Link, V. C., Stern, D. F., Weinberg, R. A., and Greene, M. I. Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies, Cell. *41:* 697-706., 1985.

5. Guy, C. T., Webster, M. A., Schaller, M., Parsons, T. J., Cardiff, R. D., and Muller, W. J. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease, Proc Natl Acad Sci U S A. *89:* 10578-82., 1992.

6. Drebin, J. A., Link, V. C., and Greene, M. I. Monoclonal antibodies reactive with distinct domains of the neu oncogene-encoded p185 molecule exert synergistic anti-tumor effects *in vivo*, Oncogene. *2:* 273-277, 1988.

7. Knutson, K. L., Hmama, Z., Herrera-Velit, P., Rochford, R., and Reiner, N. E. Lipoarabinomannan of Mycobacterium tuberculosis promotes protein tyrosine dephosphorylation and inhibition of mitogen-activated protein kinase in human mononuclear phagocytes. Role of the Src homology 2 containing tyrosine phosphatase 1, J Biol Chem. *273:* 645-52., 1998.

8. Nandan, D. and Reiner, N. E. Attenuation of gamma interferon-induced tyrosine phosphorylation in mononuclear phagocytes infected with Leishmania donovani: selective inhibition of signaling through Janus kinases and Stat1, Infect Immun. *63:* 4495-500., 1995.

9. Sorkin, A. and Waters, C. M. Endocytosis of growth factor receptors, Bioessays. *15:* 375-82., 1993.

10. Klapper, L. N., Vaisman, N., Hurwitz, E., Pinkas-Kramarski, R., Yarden, Y., and Sela, M. A subclass of tumor-inhibitory monoclonal antibodies to ErbB-2/HER2 blocks crosstalk with growth factor receptors, Oncogene. *14:* 2099-109., 1997.

11. Baselga, J., Albanell, J., Molina, M. A., and Arribas, J. Mechanism of action of trastuzumab and scientific update, Semin Oncol. *28:* 4-11, 2001.

12. Antoniotti, S., Taverna, D., Maggiora, P., Sapei, M. L., Hynes, N. E., and De Bortoli, M. Oestrogen and epidermal growth factor down-regulate erbB-2 oncogene protein expression in breast cancer cells by different mechanisms, Br J Cancer. *70:* 1095-101., 1994.

13. Baulida, J., Kraus, M. H., Alimandi, M., Di Fiore, P. P., and Carpenter, G. All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired, J Biol Chem. *271:* 5251-7., 1996.

14. Burstein, H. J., Harris, L. N., Gelman, R., Lester, S. C., Nunes, R. A., Kaelin, C. M., Parker, L. M., Ellisen, L. W., Kuter, I., Gadd, M. A., Christian, R. L., Kennedy, P. R., Borges, V. F., Bunnell, C. A., Younger, J., Smith, B. L., and Winer, E. P. Preoperative Therapy With Trastuzumab and Paclitaxel Followed by Sequential Adjuvant

Doxorubicin/Cyclophosphamide for HER2 Overexpressing Stage II or III Breast Cancer: A Pilot Study, J Clin Oncol. *21:* 46-53., 2003.

15. Jager, E., Ringhoffer, M., Altmannsberger, M., Arand, M., Karbach, J., Jager, D., Oesch, F., and Knuth, A. Immunoselection in vivo: independent loss of MHC class I and melanocyte differentiation antigen expression in metastatic melanoma, Int J Cancer. *71:* 142-7, 1997.

16. Foran, J. M., Norton, A. J., Micallef, I. N., Taussig, D. C., Amess, J. A., Rohatiner, A. Z., and Lister, T. A. Loss of CD20 expression following treatment with rituximab (chimaeric monoclonal anti-CD20): a retrospective cohort analysis, Br J Haematol. *114:* 881-3., 2001.

17. Jarvinen, T. A. H. and Liu, E. T. Novel cancer therapies targeted at the product of the HER-2/neu gene, Applied Gen Prot. *1:* 3-14, 2002.

18. zum Buschenfelde, C. M., Hermann, C., Schmidt, B., Peschel, C., and Bernhard, H. Antihuman epidermal growth factor receptor 2 (HER2) monoclonal antibody trastuzumab enhances cytolytic activity of class I-restricted HER2- specific T lymphocytes against HER2-overexpressing tumor cells, Cancer Res. *62:* 2244-7., 2002.

FIGURE LEGENDS

Fig. 1. Neu-specific monoclonal antibody treatment inhibits neu-mediated tumor cell growth *in vitro*. Shown are cell counts of neu^+ MMC (solid circles) and neu^- ANV (open circles) tumor cells grown *in vitro* with 7.16.4 antibody at concentrations of 0, 1, 10, 50, and 100μg/ml. Results are the mean of duplicate determinations expressed as a percent of control and are representative of two independent experiments yielding similar results.

Fig. 2. Neu-specific monoclonal antibody treatment, *in vitro,* does not result in the loss of cell surface neu expression. Panel A: Mean fluorescence intensity of neu expression (MFI) of control (none), non-specific IgG-treated, and 7.16.4-treated MMC. Each bar represents the mean (± s.e.m.) of 2 independent measurements using 5000 gated events. Representative histograms are shown for MMC cells treated with PBS (panel B), isotype-matched IgG (50μg/ml, panel C), and either 50 μg/ml (panel D) or 100 μg/ml (panel E) 7.16.4 antibody. Staining was performed using 7.16.4 anti-neu followed by FITC-conjugated rat anti-mouse IgG (filled histograms) or with FITC-conjugated anti-mouse IgG alone (open histograms).

Fig.3. Neu-specific monoclonal antibody inhibits neu-mediated tumor growth *in vivo* in an established tumor setting. Shown are tumor measurements from tumor-bearing control mice (PBS, circles) and tumor-bearing mice treated with 7.16.4 antibody starting on either day 10 (squares) or day 14 (diamonds) following tumor cell injection. Each data point is the mean tumor measurements (± s.e.m.) from 15-16 mice.

Fig. 4. Neu-specific monoclonal antibody treatment, *in vivo*, does not result in the loss of cell surface neu receptor expression. Panel A shows relative mean neu-specific fluorescence intensity (rMFI) of tumors from control (PBS) and 7.16.4-treated (days 10 and 14) mice. Each

bar represents the neu-specific mean rMFI (± s.e.m.) of 3 different tumors using 5000 gated events. Representative histograms are shown for PBS-treated control (panel B), and 7.16.4-treated mice with treatment started 10 days (panel C) and 14 days (panel D) after tumor cell injection. Staining was performed using 7.16.4 anti-neu followed by FITC-conjugated rat anti-mouse IgG (filled histograms) or with FITC-conjugated anti-mouse IgG alone (open histograms).

Fig. 5. Neu-specific monoclonal antibody therapy inhibits tumor development in neu-transgenic mice. Panel A shows tumor measurements from control mice (PBS, closed circles) and mice treated with 7.16.4 antibody (open circles). Each data point represents the mean (± s.e.m) of 7 mice. The arrows mark the cessation of antibody therapy. Results shown are representative of 2 independent experiments yielding similar results. Panel B shows an extended analysis of the percent of tumor free mice over a time period of 75 days following tumor injection. Treatment with 7.16.4 antibody or PBS (control) was started on day 0 and terminated on day 30 (arrow). Each symbol gives the percentage of 6 mice that were tumor free on the observation day.

Fig. 6. Neu antigen-negative variants can develop in some animals after treatment with a neu-specific antibody. Panel A shows relative mean neu-specific fluorescence intensity (rMFI) of the MMC tumor cell line (MMC), ANV tumor cell line (ANV) and 2 relapsed tumors from 7.16.4-treated mice. Each bar represents the neu-specific mean rMFI derived from a minimum of 5000 gated events. Representative histograms are shown for the MMC cell line (panel B), ANV cell line (panel C), and the relapse tumors from 7.16.4-treated mice with treatment starting at the time of tumor cell injection. Staining was performed using 7.16.4 anti-neu followed by FITC-conjugated rat anti-mouse IgG (filled histograms) or with FITC-conjugated anti-mouse IgG alone (open histograms).

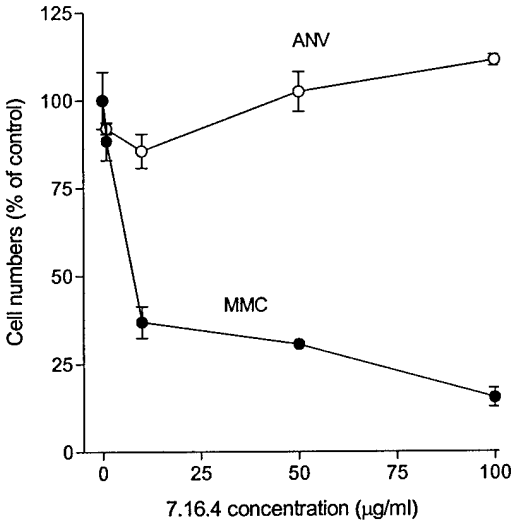
125
100
75
50
25
0
Cell numbers (% of control)
ANV
MMC
0
25
50
75
100
7.16.4 concentration (µg/ml)

Figure 1

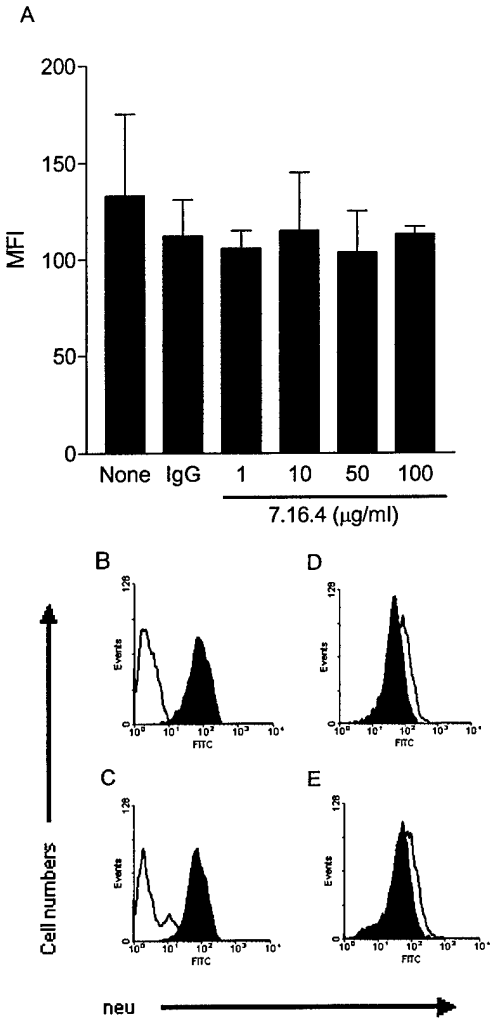
A
200
150
100
50
0
MFI
None
IgG
1
10
50
100
7.16.4 (μg/ml)
B
D
C
E
Events
FITC
Cell numbers
neu

Figure 2

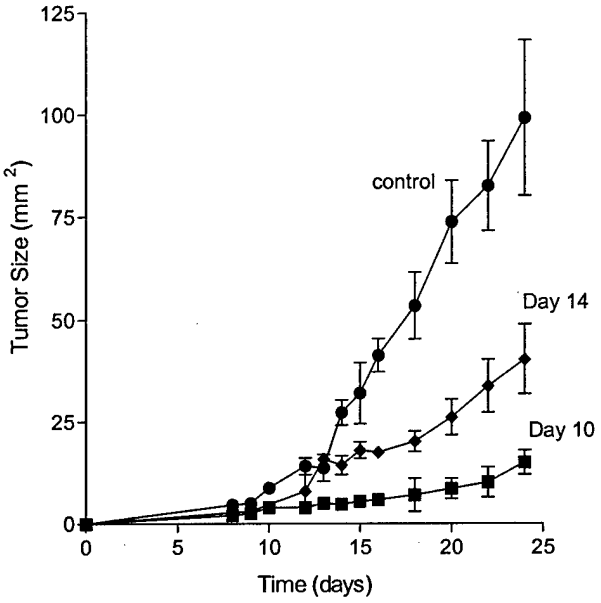
125
100
75
50
25
0
Tumor Size (mm 2)
control
Day 14
Day 10
0
5
10
15
20
25
Time (days)

Figure 3

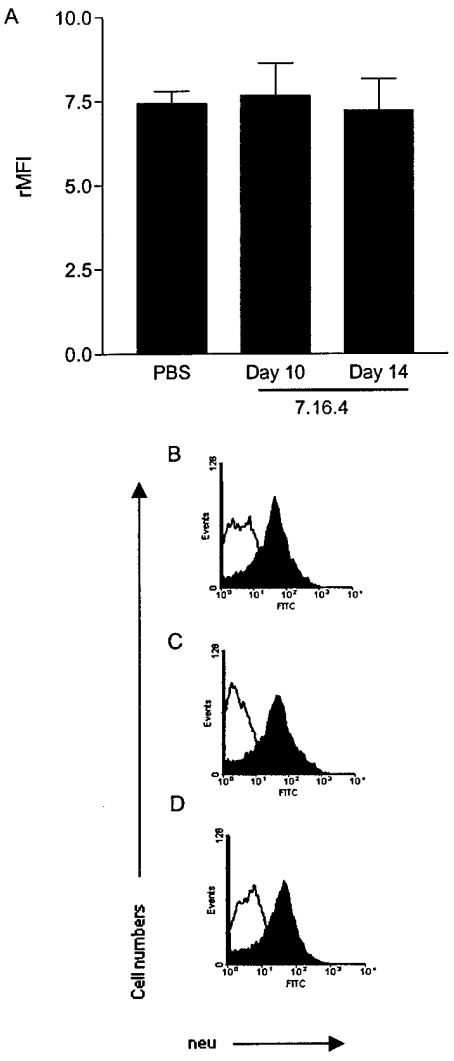
A
10.0
7.5
5.0
2.5
0.0
rMFI
PBS
Day 10
Day 14
7.16.4
B
C
D
Events
FITC
Cell numbers
neu

Figure 4

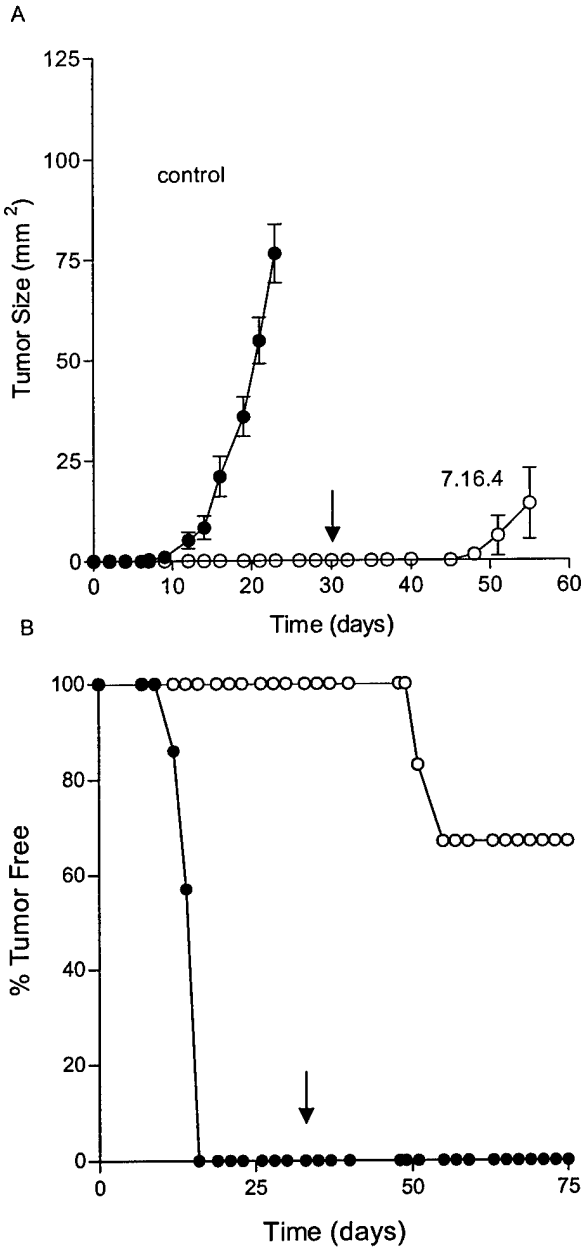
A
125
100
control
Tumor Size (mm 2)
75
50
25
7.16.4
0
0
10
20
30
40
50
60
Time (days)
B
100
80
60
40
20
0
% Tumor Free
0
25
50
75
Time (days)

Figure 5

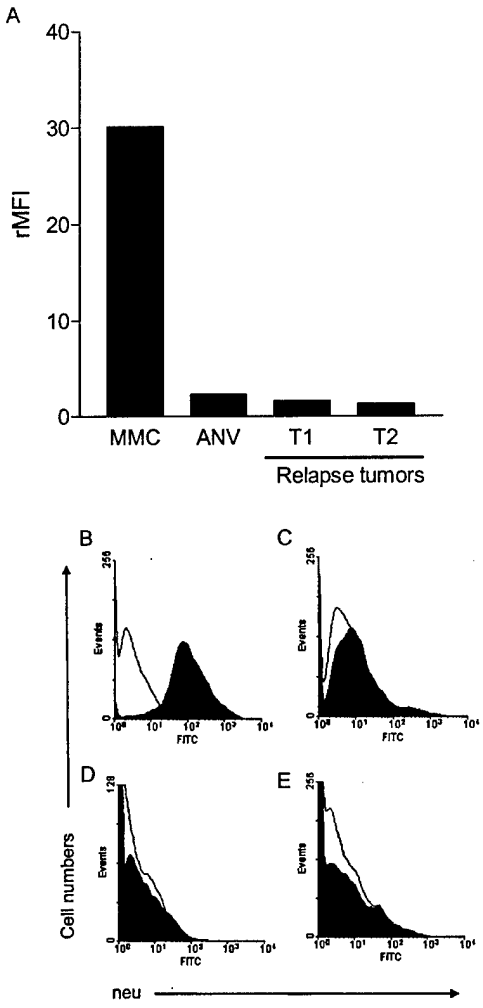
A
40
30
20
10
0
rMFI
MMC
ANV
T1
T2
Relapse tumors
B
C
D
E
Events
FITC
Cell numbers
neu

Figure 6

Immunobiol. 207, 1–8 (2003)

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Immunobiology

Soluble Cytokines Can Act as Effective Adjuvants in Plasmid DNA Vaccines Targeting Self Tumor Antigens

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Abstract

There are few vaccination strategies available for the reproducible generation of a cytotoxic T cell (CTL) response, particularly in the setting of immunizing against a tumor antigen. Plasmid-based DNA vaccination offers several advantages as compared to MHC class I peptide-based vaccines or DNA immunization using viral vectors. Plasmid-based DNA vaccines are easily produced, can potentially elicit both an MHC class I and class II response, and have little infectious potential. Plasmid-based vaccines, however, have been poorly immunogenic. The systemic immune response generated after plasmid vaccination relies on *in vivo* transfection of local antigen presenting cells (APC) and both direct presentation and "cross priming" of antigen by professional and non-professional APC. Therefore, methods to enhance the function of APC, such as simultaneous inoculation with plasmids encoding cytokine genes, has resulted in an enhancement of detectable immunity after vaccination. We questioned whether local application of soluble cytokines would be effective in enhancing the systemic immune response elicited after DNA vaccination. Using a self-tumor antigen model, we vaccinated rats with a plasmid-based rat neu intracellular domain (ICD) DNA construct and either no adjuvant, soluble GM-CSF, or IL-12. We demonstrate that the addition of soluble GM-CSF or IL-12 to rat neu ICD DNA vaccination elicits detectable neu specific T cell immunity; specifically the generation of CTL. Antibodies directed against rat neu were not elicited with this approach, indicating that the neu specific T cell immune response elicited with plasmid DNA was skewed towards cell-mediated rather than humoral immunity.

Abbreviations: APC = antigen-presenting cells; CTL = cytotoxic T cells; DC = dendritic cells; GM-CSF = granulocyte macrophage colony stimulating factor; ICD = the intracellular domain of rat neu or human HER-2/neu; id. = intradermal; IFN = interferon; IL-4 = interleukin four; IL-12 = interleukin twelve; LC = Langerhan's cells; MHC = major histocompatibility complex; Th1 = T helper cell 1 phenotype; Th2 = T helper cell 2 phenotype

Introduction

Induction of a cytolytic T lymphocyte (CTL) response requires tumor epitopes be presented via major histocompatibility (MHC) class I molecules indicating intracellular expression of tumor proteins. A common clinical method for generating tumor antigen specific CTL is to bypass the require-

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ment for intracellular processing of protein and immunize subjects with peptides designed to bind exogenously to MHC class I. Peptide-based immunization, however, has been problematic with the generation of peptide-restricted T cells that do not respond to endogenous levels of peptide presented in the MHC of tumor cells (Zaks & Rosenberg, 1998), the generation of short-lived low level immunity (Knutson et al., 2002), and the necessity of knowing the specific peptide motifs appropriate for the variety of different MHC class I molecules. An alternative method of immunization, designed to allow expression of tumor antigen proteins within the cell, is to vaccinate patients with viral vectors encoding the antigen. Endogenous expression of tumor antigen within an antigen presenting cell (APC) would result in generating T cells capable of responding to naturally processed levels of peptides, would avoid MHC restriction issues, and would allow T cell help to be stimulated via presentation of epitopes within MHC class II. However, viral based methods of immunization have been limited by the potent immunogenicity of the viral carrier making multiple immunizations problematic and posing a theoretical potential for infection in immune-compromised cancer patients. Plasmid DNA vaccines have been shown to elicit potent cell-mediated immune responses, notably with production of antigen-specific CTL and a Th1-biased phenotype of cytokine secretion (Corr et al., 1996; Raz et al., 1996; Donnelly et al., 1997; Iwasaki et al., 1997). As CTL are believed to be critical in effective antitumor immunity (Markiewicz & Gajewski, 1999), DNA-based immunization strategies are of particular interest in the development of tumor vaccines (Irvine & Restifo, 1995).

Unfortunately, the clinical translation of plasmid DNA immunization has been considerably difficult. Some studies have suggested that the immune response elicited by DNA vaccines to foreign antigens is lower in magnitude compared with protein-based vaccines and requires multiple booster immunizations (Raz et al., 1996). The low immunogenicity of DNA vaccines is a major problem when contemplating the development of cancer vaccines as the immunizing antigens are often self proteins and tolerance is a mechanism of tumor escape from immune surveillance (Nanda & Sercarz, 1995). Immune responses initiated after plasmid-based DNA vaccination are thought to be mediated by either direct transfection of APC *in vivo* or uptake of antigen via "cross presentation" by phagocytosis of non-APC transfected cells (Shedlock & Weiner, 2000). Therefore, APC, such as Langerhans cells (LC), play a major role in stimulating a systemic antigen-specific immune response after plasmid-based immunization. To that end, many groups have investigated the use of plasmid vectors encoding cytokine genes that may effect the immune microenvironment and enhance the function of local APC. The use of vectors encoding cytokine genes raises the concern of the effect of potential long-term cytokine production *in vivo*.

Local APC recruitment, maturation, and migration can be influenced by the application of soluble cytokines to the area of immunization. We have previously reported the efficacy and safety of soluble GM-CSF used as a vaccine adjuvant with peptide based vaccines both in an animal model (Disis et al., 1996) and in a human clinical trial (Knutson et al., 2001). Since plasmid vectors encoding GM-CSF and IL-12 have been shown to enhance immune responses when co-immunized with a plasmid DNA vaccine (Xiang & Ertl, 1995; Kim et al., 1997a), we questioned whether soluble cytokines could function as a vaccine adjuvant in DNA vaccines without being encoded into the plasmid. The use of soluble cytokines may circumvent the safety concerns associated with a plasmid encoding a cytokine in human clinical trials. We used a self-tumor antigen model, immunizing rats with plasmid encoding rat neu intracellular domain (ICD) and either soluble GM-CSF, or IL-12 as an adjuvant. Data presented here demonstrates vaccination with rat neu plasmid DNA and soluble GM-CSF or IL-12 given intradermally (id.) into the LC compartment, results in the generation of detectable systemic levels of neu-specific T cell immunity.

Materials and Methods

Animals

Rats used in this study were Fischer strain 344 (CDF (F-344)/CrlBR) (Charles River Laboratories, Portage MI). Animals were maintained at the University of Washington animal facilities under specific pathogen-free conditions and routinely used for experimental studies between 3 and 4 months of age.

Reagents

DNA immunization was performed with rat neu ICD inserted in pVR1012 (p-rICD), a eukaryotic expression vector (Vical, San Diego, CA). The plasmid uses kanamycin resistance for selection in *E. coli*. Plasmid DNA was purified using a Qiagen column (Valencia, CA) and was documented as

endotoxin free. Recombinant rat ICD protein was kindly provided by Dr. Paul Sleath (Corixa Corp., Seattle, WA). MATB, a Fischer rat mammary adenocarcinoma (ATCC CRL-1666) were used as a target in chromium release assays (American Type Culture Collection, Rockville, MD). K562 cells (ATCC CCL-243), used in cold target inhibition, were also obtained from ATCC.

Immunizations

Those animals who received plasmid-based vaccinations were immunized id. with 100 μg of p-rICD in PBS, with 5 μg of murine GM-CSF (Immunex Corp., Seattle, WA), or with 40 ng of murine IL-12 (R&D Systems, Minneapolis, MN). In addition, a group of control animals immunized id. with each adjuvant alone without p-rICD, GM-CSF (5 μg) and IL-12 (40 ng), were evaluated. In all groups, animals underwent 2 immunizations each 14–16 days apart. Eighteen to twenty days after the second immunization animals were assessed for immunologic response. Sera and spleens were harvested from immunized animals. Experiments included 5-animals/experimental group. Data shown here were derived from two separate immunization experiments for each group performed 2 months apart.

Evaluation of neu-specific proliferative T cell response

For analysis of neu protein-specific responses, immune spleen cells were harvested by mechanical disruption and passage through wire mesh and washed. Spleen cells (2×10^5/well) were plated into 96-well round bottom microtiter plates (Corning, Coring, NY) with 6 replicates per experimental group. The media used was EHAA 120 (Biofluids, Rockville, MD) with L-glutamine, penicillin/streptomycin, 2-mercaptoethanol, and 5% FBS. Cells were incubated with 0–2.0 μg/ml of recombinant rat neu ICD or ovalbumin as a negative control protein. After 4 days, wells were pulsed with 1 μCi of [^{3}H] thymidine for 6–8 hours and counted. Data is expressed as the mean and standard deviation cpm for each experimental group.

Evaluation of a neu-specific cytotoxic T cell response

MATB targets were prepared by infection with either wild type vaccinia virus (wtv, control) or a modified vaccinia construct containing the human ICD (v-hICD). Previous studies have shown that the vaccinated rat neu ICD response is cross reactive with human ICD (Disis et al., 1998). Five million MATB cells were incubated in media with the appropriate vaccinia construct at 5×10^6 pfu/million MATB cells along with ^{51}Cr overnight. A multiplicity of infection of 5:1 was sufficient to infect target cells and consistent expression of human ICD protein was observed in MATB after infection as determined by Western blot analysis of cell lysates (data not shown). After incubation, the labeled and infected cells were harvested and washed twice in media. Spleen cells derived from the experimental groups were incubated with the appropriate targets at a 10:1, 20:1, and 40:1 effector-to-target (E:T) ratio in quadruplicate in 96-well V-bottom plates (Corning). After 4 hours of co-incubation at 37 °C, the plates were centrifuged and 50 μl of medium from each well was assayed for ^{51}Cr content. In some experiments, K562 cells were added as cold target inhibitors at the same ratio as the MATB B targets. The % specific lysis = (sample well release-basal release)/(release due to mechanical disruption-basal release). Data is expressed as the mean and standard deviation for each experimental group.

Evaluation of neu-specific antibody response

Serologic evaluation of rat neu specific antibody immunity was performed as previously described (Disis et al., 1998). The optical density (OD) was read at 450 nm. The OD of each serum dilution was calculated as the OD of the rat neu coated wells minus the OD of the BSA coated wells. Positive control sera was derived from previous experiments where rats were immunized with human ICD protein in complete Freud's adjuvant (Disis et al., 1998).

Statistical analysis

One-sided, unpaired t tests were performed using GraphPad InStat version 3.05 for Windows 95/NT (GraphPad Software, San Diego California). p values were considered significant when < 0.05.

Results

Rat neu-specific T cell immunity could be elicited after neu DNA immunization with either GM-CSF or IL-12 as soluble adjuvants

An antigen-specific blastogenic response was assessed using thymidine incorporation assays. Animals immunized with the p-rICD without adjuvant (i.e. PBS alone) did not develop a detectable rat neu

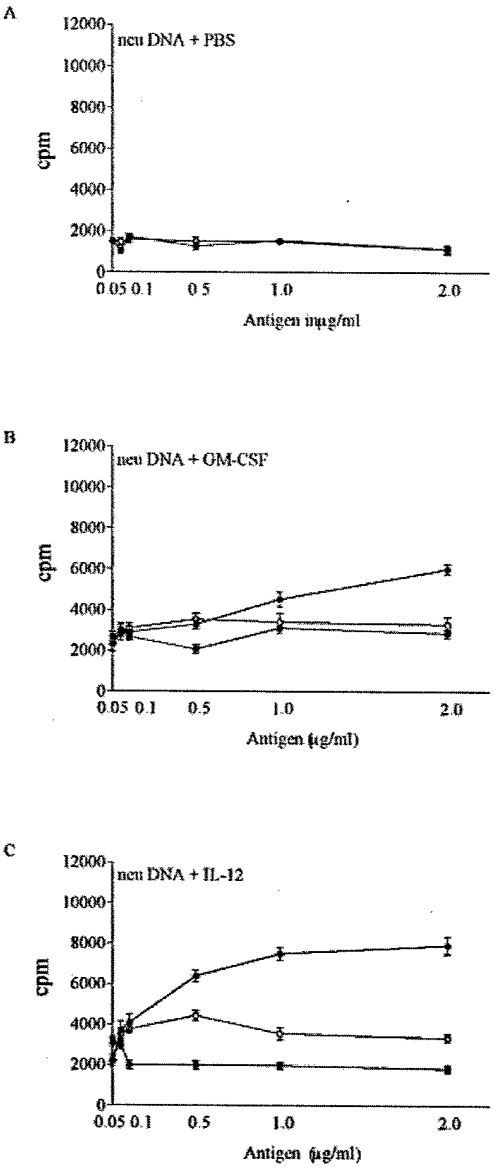

Fig. 1. Rat neu-specific T cell immunity could be elicited after neu DNA immunization with with either GM-CSF or IL-12 as soluble adjuvants. Panel A shows animals immunized with p-rICD in PBS. Panel B shows animals immunized with p-rICD and GM-CSF (●, ○) or GM-CSF alone without plasmid (■). Panel C shows animals immunized with p-rICD and IL-12 (●, ○) or IL-12 alone without plasmid (■). Closed circles in all panels represent T cell responses against experimental protein (rat neu ICD, ●) open circles depict control protein at the same concentration (ovalbumin, ○). Closed square symbol (■) represents the T cell response to rat neu ICD protein from animals immunized with cytokine alone. T cell proliferation to ovalbumin in animals immunized with a particular cytokine alone, without rat ICD plasmid, were no different from responses to rat neu ICD protein (data not shown). Data is expressed as the mean cpm and standard deviation of 5 animals per experimental group.

ICD-specific T cell response (Fig. 1A). At the most concentrated dose of antigen, 2.0 μg ICD protein, the mean cpm was 1136 ± 177 and this value did not differ significantly from the ovalbumin control response at 2.0 μg protein, 1118 ± 200 ($p = 0.42$). The mean cpm of unstimulated lymphocytes in this group was 1529 ± 111. Fig. 1B demonstrates rat ICD immunity developed when soluble GM-CSF was used as a vaccine adjuvant. Animals immunized with p-rICD and GM-CSF developed a T cell proliferative response to rat neu ICD protein at the highest concentration tested, cpm 6010 ± 101 as compared to ovalbumin 3099 ± 98 ($p < 0.01$). Animals immunized with GM-CSF alone had a mean cpm to rat neu ICD of 2872 ± 257. The mean cpm of unstimulated lymphocytes in this group was 2322 ± 215. Finally, responses elicited with IL-12 as a soluble adjuvant were also significant (Fig. 1C). Animals immunized with p-rICD and IL-12 developed a proliferative response to rat neu ICD, as example, at the highest protein concentration tested a cpm 7924 ± 320 as compared to ovalbumin 3354 ± 110 ($p < 0.01$). Animals immunized with IL-12 alone had a mean cpm to rat neu ICD of 1823 ± 190. The mean cpm of unstimulated lymphocytes in this group was 2190 ± 280.

Neu-specific CTL responses could be elicited after rat neu DNA immunization and were enhanced when GM-CSF or IL-12 were used as soluble adjuvants

The generation of CTL was assessed by chromium release assays using a syngeneic tumor cell line, MATB, infected with v-hICD or wtv, as control. Rat neu ICD and human HER-2/neu ICD are highly homologous (92% at the amino acid level) and previous studies have shown that immune responses to the ICD in rat are cross-reactive between human and rat proteins (Disis et al., 1998). Fig. 2A shows results from animals immunized with p-rICD and PBS. There was a difference in % specific lysis at the highest E:T ratio between v-hICD ($8 \pm 1\%$) infected MATB targets and those infected with wtv ($4 \pm 2\%$) ($p < 0.01$). Furthermore, neu specific CTL could be detected in animals immunized with p-rICD and soluble GM-CSF or IL-12 as adjuvant. Fig. 2B demonstrates a % specific lysis at the highest E:T ratio (40:1) of animals immunized with p-rICD and GM-CSF as a soluble adjuvant, $27 \pm 4\%$ against v-hICD MATB tumor cells. Lysis was not significantly changed by adding the cold target, K562 ($28 \pm 7\%$). Tumor cells infected with wtv demonstrated lysis at 40:1 of $8 \pm 4\%$ ($p < 0.01$). Animals immunized with cytokine alone did not lyse ICD expressing

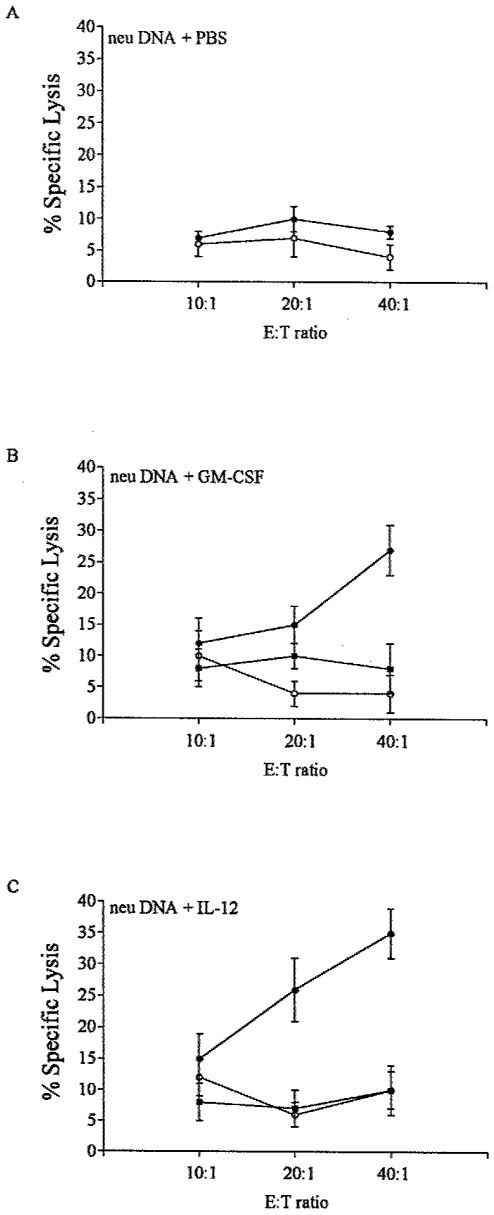

Fig. 2. Neu specific CTL responses could be elicited after rat neu DNA immunization and were enhanced when GM-CSF or IL-12 were used as soluble adjuvants. Panel A shows data from animals immunized with p-rICD in PBS. Panel B shows animals immunized with p-rICD and GM-CSF (●, ○) or GM-CSF alone without plasmid (■). Panel C shows animals immunized with p-rICD and IL-12 (●, ○) or IL-12 alone without plasmid (■). Closed circles in all panels represent CTL against ICD expressing MATB (v-hICD, ●) open circles are control MATB (wtv, ○). Closed square symbol (■) represents the T cell response v-hICD MATB from animals immunized with cytokine alone. Data is expressed as the mean and standard deviation of values obtained from 5 animals/experimental group. Repeat experiments performed 2 months later yielded similar results.

targets, (4 ± 3%). Similarly, animals immunized with p-rICD and soluble IL-12 developed neu-specific CTL. Fig. 2C demonstrates a % specific lysis at the highest E:T ratio (40:1) of 35 ± 4% against v-hICD expressing MATB tumor cells. Again, lysis was not significantly changed by adding the cold target, K562, (30 ± 6%). Tumor cells infected with wtv demonstrated lysis at 40:1 of 10 ± 3% ($p < 0.01$). Animals immunized with cytokine alone did not lyse ICD expressing targets, (10 ± 4%).

Antibody immunity to rat neu could not be detected after rat neu ICD DNA immunization

rICD-specific antibody titers were examined using ELISA. As shown in Fig. 3, neu plasmid DNA immunization was not effective in generating rat neu-specific antibody responses in any group tested. Also shown as a positive control is the antibody response to rICD that was generated in rats following immunization with hICD protein in CFA. Titers of 1:1000 yielded an OD value of 2.4 ± 0.04 (Disis et al., 1998).

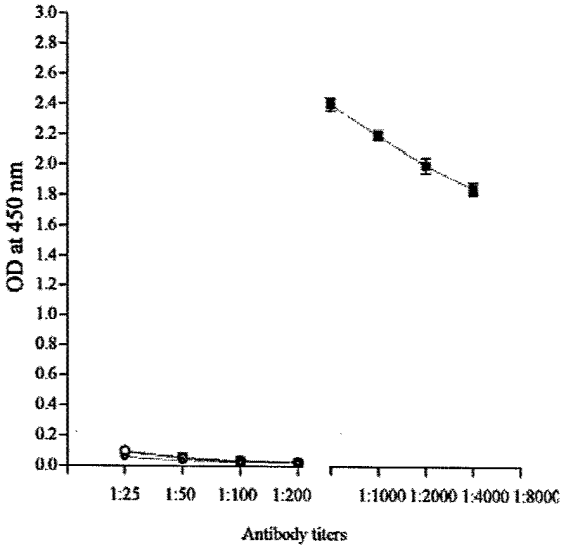

Fig. 3. Antibody immunity to rat neu could not be detected after rat neu ICD plasmid DNA immunization. Data is shown as the mean OD and standard deviation of 10 animals in each experimental group, p-rICD in PBS (◇), p-rICD with GM-CSF (○), and p-rICD with IL-12 (□). Control sera depicted with a ■ was derived from 5 rats immunized with purified human ICD protein (Disis et al., 1998) and had antibodies cross reactive with rat neu (mean and standard deviation).

Discussion

Vaccination with bacterial plasmid DNA encoding an immunogenic protein is being investigated as a means of antigen delivery due to many potential advantages (Donnelly et al., 1997). First, the approach, in contrast to peptide vaccines, can be used without knowledge of the patient MHC alleles. Secondly, using DNA-encoded antigen allows accessibility to the MHC class I processing pathway, in contrast to protein-based vaccines. Furthermore, in contrast to protein or viral vector-based vaccines, DNA is relatively easy to produce, transport, is stable in long-term storage, and can withstand fluctuations in temperature. Finally, plasmid-based strategies allow the delivery of multiple antigens simultaneously. Human cancers express multiple immunogenic proteins. The generation of antigen negative variant tumors after vaccination against a single antigen is well documented and may be circumvented by multi-antigen vaccines (Jager et al., 1997; Kerkmann-Tucek et al., 1998).

Plasmid-based DNA vaccines, however, have been only moderately successful in their translation to human clinical use. There are several reasons for the lack of success such as the low local transduction of DNA into APC as most vaccines are delivered to muscle (Gurunathan et al., 2000). Furthermore, most APC that are transduced are "non-professional" APC such as monocytes and macrophage. In addition, APC transfected *in vivo* by DNA, generally in muscle, do not home effectively to draining lymph nodes thus systemic immunity is not efficiently stimulated (Nakamura et al., 1999). Our hypothesis is that plasmid DNA immunizations may be made more effective by improving the transfection of local DC in the skin, i.e. LC, using soluble cytokines as vaccine adjuvants. Data presented here demonstrates, (1) soluble cytokine adjuvants added to plasmid-based vaccines is feasible and immunogenic, i.e. can result in circumventing tolerance to neu, a "self" tumor antigen, (2) T cell immunity could be augmented or enhanced with the use of soluble GM-CSF or IL-12 as an adjuvant, and, (3) plasmid-based DNA immunization was not effective in generating significant levels of neu specific antibodies suggesting a marked Th1 phenotypic response.

The use of cytokines as adjuvants in association with plasmid-based vaccines follows from the observation that DNA immunization elicits immune responses via presentation by professional APC (Iwasaki et al., 1997; Corr et al., 1999) and has led investigators to determine whether chemoattractant factors, or molecules that promote the differentiation of APC, might enhance the immune response elicited by DNA immunization. Several groups have demonstrated that co-immunization with plasmid DNA expressing immunostimulatory molecules can improve the efficacy of DNA vaccines. Xiang and Ertl (1995) reported that co-immunization of a plasmid vector encoding murine GM-CSF, but not IFN-γ, enhanced T cell and B cell responses to a viral antigen in a murine system. Similar results have been shown with plasmid vectors encoding IL-2 (Chow et al., 1997), IL-12 (Kim et al., 1997a), the costimulatory molecules CD80 and CD86 (Kim et al., 1997b), as well as the hematopoietic growth factor M-CSF (Kim et al., 2000). Animal models of DNA vaccines have demonstrated that immune responses can be long-lived after vaccination (Raz et al., 1994; Davis et al., 1996), likely due to persistent expression of the antigen in cells transfected *in situ* during immunization (Davis et al., 1993; Corr et al., 1996). The persistence of plasmid DNA at the site of immunization and the demonstration that plasmid DNA can be transported to distant sites by inflammatory cells (La Cava et al., 2000) raises concerns about safety when contemplating human clinical trials and at present, the long-term implications of persistent cytokine expression are unknown. The finding that soluble cytokines may play a similar role in enhancing immune responses may allow a more rapid translation of the approach to the clinic.

Soluble GM-CSF and IL-12 were both shown to promote an antigen-specific cellular immune response, and a CTL response in particular. Previous work, by our group, in the rat, demonstrated that local id. application of GM-CSF resulted in increased influx of cells expressing high levels of MHC class II into the skin and draining lymph nodes (Disis et al., 1996). Translation of this strategy into humans, in terms of a HER-2/neu peptide-based vaccine trial, demonstrated that local application of GM-CSF id. could result in persistent deposition of CD1a positive cells (LC) in the skin (Rinn et al., 1999) and the subsequent development of HER-2/neu systemic T cell immunity (Knutson et al., 2001). IL-12 is a pro-inflammatory cytokine that is secreted by activated DC and may enhance their function. Recent studies have shown that the Th1 biased responses induced by DNA vaccines are mediated by increased IL-12 production by APC (Asakura et al., 2000). Additional IL-12 in the local environment may skew that biased response further. Furthermore, IL-12, may be critical at the initiation of an immune response to promote a Th1-type of T cell differentiation with induction of antigen-specific CTL (Nakamura et al., 1997; Fallarino & Gajewski, 1999;

Murphy et al., 1999; Schmidt & Mescher, 1999). The use of IL-12 encoded within a DNA based vaccine has resulted in strongly biased Th1 response associated with a cellular, but not a humoral response (Hanlon et al., 2001) and long lasting IFNγ production but no production of IL-4 (Schultz et al., 2000). The lack of detectable neu specific antibodies in animals receiving the plasmid-based vaccine indicates a strongly skewed cell-mediated immune response.

In summary, local application of soluble cytokines known to effect APC *in vivo* is a successful strategy in generating detectable systemic immunity to a self-antigen after plasmid DNA immunization. The observation that systemic immunity was elicited implies that APC were transduced *in situ* and migrated to draining lymph nodes where antigen was presented for T cell recognition. Few immunization methods are available that effectively elicit a CTL response. Plasmid-based vaccination with soluble cytokines as adjuvant may offer an immunization strategy that is able to be rapidly applied and tested in human clinical trials of cancer vaccines.

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References

Asakura Y, Liu LJ, Shono N, Hinkula J, Kjerrstrom A, Aoki I, Okuda K, Wahren B and Fukushima J. 2000. Th1-biased immune responses induced by DNA-based immunizations are mediated via action on professional antigen-presenting cells to up- regulate IL-12 production. Clin. Exp. Immunol. 119: 130–139.

Chow YH, Huang WL, Chi WK, Chu YD and Tao MH. 1997. Improvement of hepatitis B virus DNA vaccines by plasmids coexpressing hepatitis B surface antigen and interleukin-2. J. Virol. 71: 169–178.

Corr M, Lee DJ, Carson DA and Tighe H. 1996. Gene vaccination with naked plasmid DNA: mechanism of CTL priming. J. Exp. Med. 184: 1555–1560.

Corr M, von Damm A, Lee DJ and Tighe H. 1999. *In vivo* priming by DNA injection occurs predominantly by antigen transfer. J. Immunol. 163: 4721–4727.

Davis HL, Whalen RG and Demeneix BA. 1993. Direct gene transfer into skeletal muscle *in vivo*: factors affecting efficiency of transfer and stability of expression. Hum. Gene. Ther. 4: 151–159.

Davis HL, Mancini M, Michel ML and Whalen RG. 1996. DNA-mediated immunization to hepatitis B surface antigen: longevity of primary response and effect of boost. Vaccine 14: 910–915.

Disis ML, Bernhard H, Shiota FM, Hand SL, Gralow JR, Huseby ES, Gillis S and Cheever MA. 1996. Granulocyte-macrophage colony-stimulating factor: an effective adjuvant for protein and peptide-based vaccines. Blood 88: 202–210.

Disis M, Shiota F and Cheever M. 1998. Human HER-2/neu protein immunization circumvents tolerance to rat neu: Vaccine strategy for "self" tumor antigens. Immunology 93: 192–199.

Donnelly JJ, Ulmer JB, Shiver JW and Liu MA. 1997. DNA vaccines. Annu. Rev. Immunol. 15: 617–648.

Fallarino F and Gajewski TF. 1999. Cutting edge: differentiation of antitumor CTL *in vivo* requires host expression of Stat1. J. Immunol. 163: 4109–4113.

Gurunathan S, Klinman DM and Seder RA. 2000. DNA vaccines: immunology, application, and optimization. Annu. Rev. Immunol. 18: 927–974.

Hanlon L, Argyle D, Bain D, Nicolson L, Dunham S, Golder MC, McDonald M, McGillivray C, Jarrett O, Neil JC and Onions DE. 2001. Feline leukemia virus DNA vaccine efficacy is enhanced by coadministration with interleukin-12 (IL-12) and IL-18 expression vectors. J. Virol. 75: 8424–3433.

Irvine KR and Restifo NP. 1995. The next wave of recombinant and synthetic anticancer vaccines. Semin. Cancer Biol. 6: 337–347.

Iwasaki A, Torres CA, Ohashi PS, Robinson HL and Barber BH. 1997. The dominant role of bone marrow-derived cells in CTL induction following plasmid DNA immunization at different sites. J. Immunol. 159: 11–14.

Jager E, Ringhoffer M, Altmannsberger M, Arand M, Karbach J, Jager D, Oesch F and Knuth A. 1997. Immunoselection *in vivo*: independent loss of MHC class I and melanocyte differentiation antigen expression in metastatic melanoma. Int. J. Cancer. 71: 142–147.

Kerkmann-Tucek A, Banat GA, Cochlovius B and Zoller M. 1998. Antigen loss variants of a murine renal cell carcinoma: implications for tumor vaccination. Int. J. Cancer 77: 114–122.

Kim JJ, Ayyavoo V, Bagarazzi ML, Chattergoon MA, Dang K, Wang B, Boyer JD and Weiner DB. 1997a. *In vivo* engineering of a cellular immune response by coadministration of IL-12 expression vector with a DNA immunogen. J. Immunol. 158: 816–826.

Kim JJ, Bagarazzi ML, Trivedi N, Hu Y, Kazahaya K, Wilson DM, Ciccarelli R, Chattergoon MA, Dang K, Mahalingam S, Chalian AA, Agadjanyan MG, Boyer JD, Wang B and Weiner DB. 1997b. Engineering of *in vivo* immune responses to DNA

immunization via codelivery of costimulatory molecule genes. Nat. Biotechnol. 15: 641–646.

Kim JJ, Yang JS, Lee DJ, Wilson DM, Nottingham LK, Morrison L, Tsai A, Oh J, Dang K, Dentchev T, Agadjanyan MG, Sin JI, Chalian AA and Weiner DB. 2000. Macrophage colony-stimulating factor can modulate immune responses and attract dendritic cells *in vivo*. Hum. Gene. Ther. 11: 305–321.

Knutson KL, Schiffman K and Disis ML. 2001. Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients. J. Clin. Invest. 107: 477–484.

Knutson KL, Schiffman K, Cheever MA and Disis ML. 2002. Immunization of cancer patients with a HER-2/neu, HLA-A2 peptide, p369-377, results in short-lived peptide-specific immunity. Clin. Cancer Res. 8: 1014–1018.

La Cava A, Billetta R, Gaietta G, Bonnin DB, Baird SM and Albani S. 2000. Cell-mediated DNA transport between distant inflammatory sites following intradermal DNA immunization in the presence of adjuvant. J. Immunol. 164: 1340–1345.

Markiewicz MA and Gajewski TF. 1999. The immune system as anti-tumor sentinel: molecular requirements for an anti-tumor immune response. Crit. Rev. Oncog. 10: 247–260.

Murphy KM, Ouyang W, Szabo SJ, Jacobson NG, Guler ML, Gorham JD, Gubler U and Murphy TL. 1999. T helper differentiation proceeds through Stat1-dependent, Stat4-dependent and Stat4-independent phases. Curr. Top. Microbiol. Immunol. 238: 13–26.

Nakamura T, Lee RK, Nam SY, Podack ER, Bottomly K and Flavell RA. 1997. Roles of IL-4 and IFN-gamma in stabilizing the T helper cell type 1 and 2 phenotype. J. Immunol. 158: 2648–2653.

Nakamura K, Saitoh A, Yasaka N, Furue M and Tamaki K. 1999. Molecular mechanisms involved in the migration of epidermal dendritic cells in the skin. J. Investig. Dermatol. Symp. Proc. 4: 169–172.

Nanda NK and Sercarz EE. 1995. Induction of anti-self-immunity to cure cancer. Cell 82: 13–17.

Raz E, Carson DA, Parker SE, Parr TB, Abai AM, Aichinger G, Gromkowski SH, Singh M, Lew D, Yankauckas MA, et al. 1994. Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. Proc. Natl. Acad. Sci. USA 91: 9519–9523.

Raz E, Tighe H, Sato Y, Corr M, Dudler JA, Roman M, Swain SL, Spiegelberg HL and Carson DA. 1996. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. Proc. Natl. Acad. Sci. USA 93: 5141–5145.

Rinn K, Schiffman K, Otero HO and Disis ML. 1999. Antigen-specific recall urticaria to a peptide-based vaccine. J. Allergy. Clin. Immunol. 104: 240–242.

Schmidt C and Mescher M. 1999. Adjuvant effect of IL-12: Conversion of peptide antigen adminstration from tolerizing to immunizing for CD8+T cells *in vivo*. J. Immunol. 163: 2561–2567.

Schultz J, Heinzerling L, Pavlovic J and Moelling K. 2000. Induction of long-lasting cytokine effect by injection of IL-12 encoding plasmid DNA. Cancer Gene Ther. 7: 1557–1565.

Shedlock DJ and Weiner DB. 2000. DNA vaccination: antigen presentation and the induction of immunity. J. Leukoc. Biol. 68: 793–806.

Xiang Z and Ertl HC. 1995. Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. Immunity 2: 129–135.

Zaks TZ and Rosenberg SA 1998. Immunization with a peptide epitope (p369-377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu+tumors. Cancer Res. 58: 4902–4908.

Expert Opinion

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Adoptive T-cell therapy for the treatment of solid tumours

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Solid tumours can be eradicated by infusion of large amounts of tumour-specific T-cells in animal models. The successes seen in preclinical models, however, have not been adequately translated to human disease due, in part, to the inability to expand tumour antigen-specific T-cells *ex vivo*. Polyclonality and retention of antigen-specificity are two important properties of infused T-cells that are necessary for successful eradication of tumours. Investigators are beginning to evaluate the impact of attempting to reconstitute full T-cell immunity representing both major T-cell subsets, cytolytic T-cells and T-helper (Th) cells. One of the more important and often overlooked steps of successful adoptive T-cell therapy is the *ex vivo* expansion conditions, which can dramatically alter the phenotype of the T-cell. A number of cytokines and other soluble activation factors that have been characterised over the last decade are now available to supplement *in vitro* antigen presentation and IL-2. Newer molecular techniques have been developed and are aimed at genetically altering the characteristics of T-cells including their antigen-specificity and growth *in vivo*. In addition, advanced imaging techniques, such as positron emission tomography (PET), are being implemented in order to better define the *in vivo* function of *ex vivo* expanded tumour-specific T-cells.

Keywords: cytotoxic T-cells (CTL), cancer, cytokine, dendritic cells, *ex vivo* expansion, T-helper cell, polyclonal, T-cell receptor, vaccine

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1. Introduction

Animal models demonstrate that adoptive T-cell therapy of advanced stage malignant disease is a feasible and successful treatment strategy. Increased understanding of the complex nature of the immune effector cells and the identification of tumour antigens is providing researchers with the appropriate tools to generate and reconstitute effective tumour-specific immunity through adoptive transfer of T-cells. *Ex vivo* expansion has been problematic and many hurdles will need to be overcome. Two advances in T-cell culture have improved the ability to generate tumour-specific T-cells *ex vivo*. The first was to increase antigen-primed T-cells *in vivo* prior to *ex vivo* expansion by active immunisation. The second was the improvement of culture conditions with the use of recently identified tumour antigens, cytokines and co-stimulatory molecules in conjunction with IL-2. In addition, technologies are being developed to genetically modify T-cells to create the appropriate immune microenvironment for tumour destruction. A better understanding of immunoregulatory mechanisms will allow us to overcome tumour-induced immunosuppression in cancer patients during adoptive T-cell therapy.

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2. The clinical role of adoptive T-cell therapy

Adoptive T-cell therapy is an immune-based therapeutic strategy that will significantly boost tumour-specific T-cell immunity above that observed by vaccination alone [1]. Transferred T-cells can potentially represent a major fraction (1:2) of the host's lymphocytes [1]. This strategy could have an advantage over active vaccination for the treatment of more extensive malignancy where greater control of the numbers of tumour-specific T-cells may be required. Adoptive T-cell therapy techniques that have shown great promise in human clinical trials have been applied to the Epstein-Barr virus (EBV)-related disorders, immunoblastic lymphoma and Hodgkin's disease [2-4]. These studies, as will be further discussed, provide proof of principle that administration of *ex vivo* expanded T-cells can reconstitute effective long lasting immunity *in vivo*. However, the major distinction between the treatment of EBV-malignancies and non-viral malignancies is the targeting of non-self viral antigens. In the most common solid tumours, self proteins are the target antigens of T-cells. Thus, *ex vivo* expansion of these T-cells is further complicated by the probability that the most potent and robust self tumour antigen-specific T-cells recognising self antigens have either been deleted or rendered ineffective by tolerising mechanisms [5,6]. *Ex vivo* expansion methods must be optimised to maximise functional effects of the remaining self-reactive T-cells while preventing the outgrowth of non-specific immune effector cells. Recent evidence demonstrating that single clones are ineffective at mediating tumour eradication further suggests that *ex vivo* expansion methods should also be designed to maintain polyclonality in order to ensure multiple specificities for the same epitope as well as multiple T-cells subsets specific for the target antigen [7]. The need for polyclonal responses is particularly important for a number of reasons, including the prevention of antigen-loss variants, the prevention of major histocompatibility complex (MHC) class I loss variants and the need of helper activity by cytotoxic T-cells (CTL) for expansion and persistence *in vivo*. Much of our current thinking of the obstacles of adoptive T-cell therapy stems from mouse models and the results of previously reported human clinical trials.

3. Human clinical trials of adoptive T-cell therapy

Clinical trials of adoptive T-cell therapy arose from promising earlier studies evaluating the antitumour efficacy of lymphokine activated killers (LAK). LAK are generated from patient peripheral blood mononuclear cells (PBMC) with IL-2 and are capable of killing tumour cells in a non-MHC-restricted fashion. Clinical trials, utilising LAK, have been carried out by several groups treating a variety of carcinomas, including melanoma, ovarian, renal cell and colorectal [8-10]. The low response rates ranging from 0 - 20% following infusion of LAK are likely a reflection of the inability of the LAK to home to tumour sites and the lack of specificity for the tumour. However, the response rates were encouraging and provided impetus for identifying lymphocyte populations, mainly T-cells, that have increased specificity and antitumour effector function.

The strategies which have been developed for increasing the number of tumour-specific T-cells, *ex vivo*, have included the non-specific expansion of tumour infiltrating lymphocytes (TIL), the non-specific expansion of sensitised lymph nodes draining the tumour site and the expansion of tumour antigen-specific T-cell populations. The first two strategies are based on the hypothesis that there is an increased precursor frequency of tumour-specific T-cells at sites local to the tumour or in the lymph node draining the tumour. The last strategy exploits the use of known tumour antigens to expand specific T-cell populations.

Beginning in the late 1980s, clinical trials were carried out using *ex vivo* expanded TIL from a variety of cancers including malignant melanoma, renal cell carcinoma, breast cancer and lung cancer. Response rates after TIL therapy were variable and ranged between 0 - 60% with most being between 10 - 25% [11]. Studies using TIL have been difficult to perform in cancer patients due to:

- The limitations in obtaining significant amounts of tumour from which to derive the cells.
- The inability to expand autologous cancer target cell lines to test T-cell lytic activity prior to infusion.
- The location of metastatic relapse (e.g., bone, lung, brain and liver) prevents tumour cell and thus T-cell harvest.

TIL are typically expanded by incubating the cells with very high concentrations, up to 7000 U/ml, of IL-2. These culture conditions typically result in enriching for T-lymphocytes with LAK-like activity. For example, Belldegrun and colleagues reported the results of a study characterising the cell populations derived from *ex vivo* expansion of lymphocytes infiltrating human renal cell cancer [12]. Renal TIL expanded in high doses of IL-2 are predominantly CD3+ but display lytic activity similar to LAK, including high activity against K562, Daudi and allogeneic tumour cells. Ratto and colleagues observed similar findings following expansion of lung TIL [13].

The use of IL-2-expanded TIL in melanoma trials has led to observations that could have important implications for the design of future adoptive T-cell trials. Rosenberg and colleagues reported the results of a clinical trial evaluating treatment of melanoma patients with both TIL and high dose bolus IL-2 [14,15]. Of the 86 patients treated, 24 partial and five complete responses were observed. A number of important correlations were observed when comparing the *in vitro* characteristics of the expanded TIL of responders with the TIL of the non-responders. Clinical responses were associated *in vitro* cellular responses such as higher specific lysis of autologous tumour targets, shorter doubling times, younger cul-

tures and increased autologous tumour-specific granulocyte-macrophage colony stimulating factor (GM-CSF) secretion [15].

Like TIL, lymph nodes draining either a tumour or vaccine site represent another potentially rich source of tumour-specific T-cells. Clinical response rates following reinfusion of *ex vivo* activated nodal T-cells are similar to TIL infusions [11]. In a recent Phase I study, To and colleagues evaluated the toxicity and clinical responses of infusions of *ex vivo* expanded vaccine-draining lymph node-derived lymphocytes in patients with head and neck cancers [16]. Fifteen patients were vaccinated on the thigh with irradiated autologous tumour with GM-CSF. After 8 - 10 days, the inguinal, vaccine-draining lymph nodes were harvested and activated *ex vivo* with staphylococcal enterotoxin A (SEA) and expanded in high dose IL-2. The resulting cells were mainly CD3+ and had mixed CD8+ and CD4+ phenotype. Toxicity following reinfusion was minimal and limited to grade 2. Of the 15 patients, only two responded with one patient being disease free. In a similar study, patients with newly diagnosed gliomas were immunised with autologous tumour cells followed by harvesting and expansion of the vaccine-draining lymph nodes [17]. The trial resulted in four partial responses out of 12 patients treated. Further *in vitro* characterisation of the resulting T-cell populations can provide more insight into how this promising technique can be optimised. As an example, perhaps increased tumour killing could be achieved by including specific tumour antigens during expansion.

Recent advances in molecular and cellular immunology provide the technology needed to identify and define tumour-specific antigens, as well as an understanding of how T-cells recognise antigens. It is presumed that a highly purified, antigen-specific and polyclonal T-cell population would be the most efficient strategy for tumour eradication. Clinical trials testing the feasibility of antigen-specific T-cell therapy for the treatment of disorders and cancers associated with EBV have been reported. EBV-specific CTL lines have been generated *in vitro* from donor blood and used successfully to treat B-cell lymphoproliferative disorder in bone marrow transplant recipients [4]. In addition, EBV-specific CTL have been expanded *ex vivo* and reinfused into patients with advanced stage relapsed Hodgkin's disease [2]. This feasibility study demonstrated:

- CTL could be expanded from patients with advanced cancer.
- T-cells were found to persist up to 13 weeks after infusion.
- Antigen-specific T-cells were detected in the pleura at levels 10-fold higher than the peripheral blood, implying trafficking of infused T-cells to sites of tumours.
- Transferred T-cells produced increased EBV-specific cytotoxic activity in peripheral blood lymphocytes as measured by chromium release assay and a decrease in peripheral blood viral burden as measured by quantitative PCR.

A preliminary report of the clinical status of patients receiving this treatment indicated that in addition to reducing viral burden, the T-cell infusion may stabilise or reduce disease symptoms [18] suggesting that Phase II clinical trials should be carried out to evaluate clinical responses.

Recently, Dudley and colleagues assessed the safety, feasibility and clinical response of adoptive T-cell therapy of melanoma using *ex vivo* expanded CD8+ cytolytic T-cell clones specific for an HLA-A2 binding peptide derived from the melanoma antigen GP-100 [7]. Twelve patients were treated with multiple infusions of GP-100-specific T-cell clones with an average of 1 x 10^{10} cells/infusion. The T-cells clones were selected based on their apparent avidity for peptide antigen as assessed by the magnitude of antigen-specific cytokine release. Although the clones secreted large amounts of IFN-γ and recognised HLA-A2+ melanoma cell lines, only two patients had minor partial responses. An important finding from this study was that the T-cells disappeared rapidly and were undetectable at 2 weeks, even though the patients received concomitant iv. IL-2. The authors postulated several mechanisms to explain the lack of clinical effects. One possibility is that the CD8+ T-cell clones could not persist in the absence of sufficient help, and the authors suggested that the treatment could be improved by co-infusion with antigen-specific CD4+ T-cells. This is supported by previous studies by Walter and colleagues who observed that CMV-specific CTL clones declined more rapidly in patients deficient in CD4+ Th cells specific for CMV [19].

These clinical trials as well as many others identify major areas that should be further investigated to improve the clinical outcome of adoptive immunotherapy. Namely, identification of *ex vivo* expansion conditions and other novel molecular techniques aimed at improving the antigen-specificity, functionality, polyclonality and longevity of the infused T-cells.

4. *Ex vivo* expansion of tumour-specificT-cells

The success in expanding EBV-specific T-cells from peripheral blood of patients may be related to the endogenous, naturally occurring high precursor frequency of these cells, thus, the abundance of viral-specific T-cells in the initial cultures. In contrast to viral cancers, T-cells directed against non-viral tumours are much less abundant [20]. *Ex vivo* expansion of tumour antigen-specific T-cells may be greatly facilitated by prior immunisation against specific tumour antigens. The authors recently demonstrated the feasibility of this approach in breast and ovarian cancer patients who were vaccinated against HER-2/neu with a helper peptide-based vaccine [21]. Nineteen patients were vaccinated with a HER-2/neu peptide-based vaccine consisting of three helper peptides, each of which contained an HLA-A2 binding motif, fully nested within its sequence. Six monthly vaccinations with GM-CSF as adjuvant resulted in increased levels of T-cells specific for the encompassed HLA-A2 motifs to levels similar to those measured against influenza and CMV HLA-A2-binding peptides. The increased precursor frequency after vaccination improved the generation of T-cell clones specific for the HER-2/neu HLA-A2 binding peptide, p369-377. As an

example, in one patient, a total of 21 p369-377 clones were generated. With the exception of two clones, all clones were CD3+ [22]. Eleven of the clones were CD8+/CD4–. Nine of the clones were CD4+/CD8-, despite being specific for an HLA-A2 binding peptide. The remaining five clones contained varying levels of both CD4+ and CD8+. The majority (19/21) of clones expressed the α/β T-cell receptor but interestingly, two clones expressed the γ/δ T-cell receptor [23]. Several of these clones could be induced to secrete IFN-γ in response to p369-377 peptide stimulation. Several clones were able to lyse HLA-A2-transfected HER-2/neu-overexpressing tumour cells, including the γ/δ TCR expressing clones. Similarly, Reddish and colleagues demonstrated that breast cancer patients can generate MHC class I-restricted CTL against MUC-1-expressing adenocarcinomas following vaccination with a MUC-1 helper peptide [24]. Investigations such as these demonstrate that *ex vivo* expansion and characterisation of cancer specific T-cells is facilitated by vaccination and that the responses elicited to vaccine can be diverse and polyclonal.

Several techniques for *ex vivo* expansion of tumour-antigen T-cells are currently being developed in various laboratories. Two variables that can be manipulated during *ex vivo* expansion are the antigen and cytokine environments. The identification of tumour-specific antigens, as well as the important tumour-responding T-cell populations, has ushered in a new era of cellular expansion techniques that can generate T-cell lines and clones with increased antigen-specificity. Manipulating the cytokine environment also allows for the preferential expansion of T-cell subsets such as CD4+ and CD8+ or Th1 and Th2. Antigen-specific techniques are preferable over non-antigen-specific techniques due to the fact that even with vaccination, antigen-specific T-cell precursors may not be at sufficient levels to expand preferentially during stimulation with non-specific activation such as with anti-CD3/anti-CD28 beads or bacterial products such as SEA. This is evident from previous studies demonstrating that expansion of tumour-infiltrating T-cells with non-specific methods does not promote expansion of tumour-specific T-cells that are therapeutic *in vivo* [16,17]. These methods may activate all T-cells to a similar degree and may result in the expansion of non-specific bystander T-cells, immunosuppressive T-cells, or tolerated, non-functional T-cells. Methods are now being established allowing for selective expansion of specific T-cell subsets.

Adoptive T-cell therapy strategies have largely focused on the *ex vivo* generation of CTL due to observations that most tumours express MHC class I but not MHC class II and that CTL can mediate tumour regression in mice. Studies demonstrating the weak persistence of transferred CTL have led to investigations on how to extend their lifespans. Recent evidence from our laboratory suggests that simultaneous generation of tumour antigen-specific CD4+ Th cells could prolong the life of CTL *in vivo* [21]. The authors observed that > 60% of patients immunised with HER-2/neu helper epitopes, each containing an encompassed HLA-A2 epitope, were able to develop HER-2/neu specific CD8+ T-cell immunity. The CD8+ T-cell response was maintained, in some patients, for at least one year following vaccination. In contrast, 2/5 (40%) patients immunised with a single HER-2/neu HLA-A2 9-mer peptide, p369-377 (E75) developed HER-2/neu CD8 T-cell immunity that declined to undetectable levels within 5 months of the last vaccination (Knutson & Disis, unpublished observations). These data are consistent with findings in murine viral models where persistence of CD8+ T-cells is critically dependent on concurrent CD4+ T-cell immunity [25]. For example, in the murine LCMV model of viral immunity, loss of CD4 T-cell help results in impaired memory phase CTL responses leading to the inability of the mice to permanently control infection [26-28]. Moreover, CD4+ T-cells may also possess direct and indirect killing properties [29]. CD4+ T-cells encountering tumour directly or indirectly through dendritic cell (DC) cross-presentation can release a wide variety of cytokines, such as TNF-related apoptosis-inducing ligand (TRAIL), which can activate apoptotic pathways in tumour cells [30,31].

The *ex vivo* expansion of tumour antigen-specific CD4+ T-cells has been impeded by the lack of defined MHC class II-restricted tumour antigen peptides and the appropriate cytokine environment optimal for the generation of CD4+ T-cells capable of eliciting an inflammatory or Th1 type response. The authors had previously identified several MHC class II peptides, derived from HER-2/neu [32]. It was determined which of these peptides were relevant immunogens based on their ability to induce T-cells that recognised naturally processed HER-2/neu protein antigens in breast, ovarian and lung cancer patients [33]. Thus, with the identification of relevant MHC class II antigens, an important objective was to define the appropriate cytokine environment that preferentially promotes the expansion of HER-2/neu-specific CD4+ T-cells with a Th1 type profile, a phenotype important to the development of a cell-mediated immune response at the tumour site. Th1 cells could be efficiently cultured by the inclusion of IL-12 along with peptide and IL-2 during culture. IL-12 is a heterodimeric cytokine, produced by B-cells, macrophages and professional antigen presenting cells (APC), that has multiple effects on CD8 T-cell function when added together with low-dose IL-2 [34-39]. As a model MHC class II antigen, the authors chose p776-790 derived from the intracellular domain of HER-2/neu. The majority of patients immunised with this peptide developed immunity to HER-2/neu protein. Furthermore, this epitope was commonly associated with epitope spreading suggesting natural presentation [40]. While immunity to p776-790 could be readily measured in short-term cultures, cell lines generated by *in vitro* stimulation with peptide and IL-2 as the only added cytokine resulted in no antigen-specific expansion. The inclusion of IL-12, along with IL-2, restored antigen-specific responsiveness in a dose-dependent fashion [41]. The resulting p776-790-specific T-cells responded readily to antigen by proliferating and producing Type I cytokines (IFN-γ and TNF-α). The

increased proliferative response of the cultures was due in part to an increase in the number of HER-2/neu-specific T-cells as assessed directly by ELIspot analysis. Inclusion of IL-12 into the cultures also resulted in a significant decrease of non-specific cellular proliferation. These results suggest that IL-12 is an important cytokine for *ex vivo* recovery and maintenance of antigen-specific CD4+ T-lymphocytes that would otherwise be lost by using IL-2 as the only cytokine.

IL-7 has shown promise for the expansion of CTL under certain *ex vivo* conditions. IL-7 is a stromal cell-derived cytokine and is associated with the early development of lymphoid cells. IL-7 activates the proliferation of naive T-cells and has been implicated as a key cytokine in maintaining homeostatic proliferation *in vivo* [42]. Recent findings suggest that the addition of IL-7 to cultures can promote preferential expansion of antigen-specific T-cells [43]. TIL derived from follicular lymphoma (FL) typically lack tumour-specific activity which is not recovered by culturing cells with FL along with IL-2. However, when TIL are preactivated through CD40 followed by exposure to FL they can be further expanded by inclusion of IL-7 along with IL-2. The expanded T-cells have greatly enhanced FL-specific CTL activity. The effects of IL-7 appear however to depend on the *ex vivo* expansion environment. In the lab the authors have found that when IL-7 is included along with an influenza matrix peptide and IL-2, peptide-specific lysis is reduced by 25 - 30% compared to cells cultured with peptide and IL-2 alone (Knutson, unpublished observations). The background, non-specific lysis was also increased 3-fold. This lack of effect of induction of peptide-specific responses may not be translatable to other peptide systems. For example, Tsai and colleagues have found that IL-7 potentiated the ability of peptide-pulsed DC to generate CTL responses against viral and tumour epitopes [44]. These discrepancies in outcome clearly point to the need to optimise the use of cytokines in preclinical studies prior to clinical trials.

Recent studies with IL-15 demonstrate that this cytokine can have important effects on *ex vivo* expansion of peptide- and protein-specific T-cells. IL-15 is structurally similar to IL-2 and their receptors share the IL-2Rβ and IL-2γ chains [45]. The IL-2Rα (CD25) and IL-15Rα chains confer specificity. Like IL-2, IL-15 is a pleiotropic cytokine and induces proliferation and functional changes of multiple haematopoietic cells including αβT-cells, γδT-cells, DC and NK cells [46]. IL-2 predisposes T-cells to undergo activation-induced cell death (AICD) and IL-15 promotes the generation of memory CD8+ T-cells [47]. The death-inducing effects of IL-2 are particularly important for the expansion of CD4+ T-cells which are extremely sensitive to IL-2 following antigen stimulation. IL-2 and IL-15 also can change the homing capabilities of cultured CD8+ T-cells [48]. Antigen-primed murine CD8+ T-cells cultured in IL-15 but not IL-2, preferentially home to lymphoid tissue such as spleen and lymph nodes, while IL-2 cultured CD8+ T-cells home to sites of inflammation but not lymphoid tissue. IL-15 cultured cells home to sites of inflammation to a lesser extent but mediate a robust antigen recall response. Currently it is unknown if these findings can be extrapolated to human T-cells but could have important implications in designing expansion conditions to generate T-cells capable of targeting lymph node disease. Pharmacological generation of an inflammatory response with anti-apoptotic agents may be considered in order to attract IL-2-cultured cells to tumour site.

Activation of T-cells directly through CD40 or indirectly using trimeric CD40L also enhances the expansion of antigen-specific T-cells [49]. CD40L is expressed on T-cells and is the ligand for CD40 which is expressed on antigen-presenting cells, including DC [50]. The interaction of CD40 with CD40L is IL-2- and CD28- independent and results in increased proliferation of T-cells as well as increased expression of Th1 cytokines (IFN-γ, TNF-α) [49]. During induction of tumour-specific immunity, CD40 activation promotes long-term survival of tumour-specific CD8+ T-cells [51]. Terheyden and colleagues have recently reported encouraging results demonstrating the utility of CD40 ligation during *ex vivo* expansion of melanoma-specific CD8+ T-cells and CD4 T+ cells derived from TIL [52]. When T-cells were cloned from TIL using oncolysate-pulsed DC, the resulting phenotype of the cell population was predominantly CD4+ Th2-like cells demonstrating high IL-4 production. The inclusion of anti-CD40 monoclonal antibody (mAb) to ligate CD40 on autologous DC induced both CD8+ and CD4+ T-cells that were specific for melanoma antigens. The CTL had high lytic activity against autologous tumour but not allogeneic tumour nor autologous fibroblasts. While many of the CD4+ T-cell clones were exclusively Th1 (IFN-γ+, IL-4-), many possessed a Th0 (IFN-γ+, IL-4+) phenotype. The clinical significance of these latter Th0 cells are currently unknown but newer findings in murine models of adoptive T-cells therapy suggest that better tumour eradication is achieved with both cell-mediated and humoral immunity [53].

DC-based T-cell expansion methods are an attractive approach because DC produce many of the necessary soluble activation factors for *ex vivo* expansion of antigen-specific T-cells. Several DC-based strategies are currently being developed for expansion including loading DC with apoptotic tumour cells, tumour antigen peptides, protein, or tumour cell-derived RNA. DC are the most potent antigen-presenting cells of the immune system and are responsible for initiating and modulating the immune response [54]. Unlike tumour cells, DC express co-stimulatory molecules and MHC class I and class II at high levels, and only a small number of DC are required to activate and expand tumour-specific T-cells.

The frequency of restimulation as well as the source of antigen appear to be important for determining the phenotype of the resulting T-cell population when using DC-based expansion. While repeated weekly stimulation of PBMC with renal cell carcinoma-loaded DC resulted in a predominant expansion of CD4+ T-cells, alternating between tumour cell loaded DC and irradiated tumour cells alone resulted in enriched

CD8+ T-cells with potent specificity for renal cell lines [55]. Preferential expansion of tumour-specific CD8+ T-cells can be accomplished by prior enrichment with reagents such as peptide MHC class I tetramers [56] before stimulation with tumour cell- or antigen-pulsed DC. DC can also be transfected with tumour-derived RNA as has recently been reported by Heiser and colleagues for the *ex vivo* generation of polyclonal T-cells against prostate and renal cell carcinoma [57,58].

Tumour cell or tumour RNA isolation can be avoided by transfecting or transducing DC with whole tumour antigen DNA, an approach that is attractive because it obviates the need to tailor make peptide antigens specific for the patient's MHC haplotype. This approach has been used by Bushenfelde and colleagues to generate CTL and Th cells specific for HER-2/neu using a retroviral transduction of DC [59]. DC were generated from CD34+ stem cells derived from patients and were transduced to express the full length HER-2/neu. Patient PBMC were then stimulated with the HER-2/neu-expressing DC. After 3 weekly stimulations, both CTL and Th cells could be individually cloned. The clones recognised different regions of the HER-2/neu suggesting the capacity of the DC to express multiple antigenic peptides. An important attribute of this approach is that the transduced DC do not express high levels of HER-2/neu but rather moderate to low levels, which would support the generation of high affinityT-cells which would in turn, be able to target tumour cells with a broad range of antigen expression [59].

While *ex vivo* expansion techniques can be optimised to produce T-cell lines with improved antigen-specificity and function, as assessed by *in vitro* assays, the ultimate goal is to carry those attributes into the host following transfer. The complex *in vivo* environment can present new challenges to the T-cells that may not have been present *in vitro*. For example, the immune microenvironment contains immunosuppressive factors which will inhibit the growth and function of transferred cells. Methods are being developed to improve the function of T-cells *in vivo* and to overcome these obstacles.

5. Enhancing *in vivo* function and longevity of transferred T-cells

In vivo supplementation with soluble T-cell growth factors can promote the activation and longevity of adoptive transferred T-cells. For example, murine models of adoptive T-cell transfer have demonstrated that administration of IL-2 following adoptive transfer maintains high levels of precursor specific for viral antigens for extended periods of time [1]. This is consistent with studies in IL-2 knockout mice where frequencies of transferred ovalbumin (OVA)-specific CD8+ T-cells declined significantly with time compared to the same T-cells injected into normal mice [60]. However, the toxicity of IL-2 in cancer patients limits its use and investigations are underway to identify less toxic strategies for the chronic maintenance of transferred T-cells. Recent findings suggest that iv. IL-2 alone may not be sufficient to extend the life of transferred melanoma-specific CD8+ T-cell clones, suggesting the need for additional factors [7]. Recent findings by the authors demonstrate that HER-2/neu-specific CD8+ T-cells can persist for at least a year when generated concurrently with HER-2/neu-specific CD4+ T-cells suggesting that co-infusion of antigen-specific CD4+ T-cells along with CD8+ T-cells during adoptive T-cell therapy may improve CD8+ T-cell longevity [21]. Alternatively, it would be beneficial to identify other pharmacological means of improving function and extending T-cell life *in vivo*.

One candidate receptor for *in vivo* modulation of infused T-cells is the OX-40 receptor (OX-40R). OX-40R is a transmembrane receptor expressed predominantly on activated CD4+ T-cells and is a member of the TNF receptor superfamily. The ligand for OX-40, OX-40L, is expressed on activated APC and B-cells. *In vivo* engagement of OX-40R with OX-40L during tumour priming results in enhanced tumour immunity through increased activation of the endogenous antitumour CD4+ T-cell response [61,62]. These responses were observed for a variety of murine tumours. Recently, OX-40R activation using OX-40 mAb has been applied to adoptive T-cell therapy of 10-day MC205 pulmonary metastases and intracranial tumours. In that study it was found that administration of OX-40R mAb resulted in the need for significantly fewer tumour-specific T-cells to cure established malignancy [63]. *In vivo* T-cell trafficking studies revealed that the OX-40R mAb application did not result in an increased number of T-cells trafficking to tumour sites suggesting that OX-40R stimulation results in enhanced function of tumour-specific T-cells.

Methods of genetic modification of T-cells to enhance cellular function, deliver therapeutic factors, or enhance T-cell longevity will likely play a key role in the success of adoptive T-cell therapy in tumour eradication [64]. The feasibility of these approaches has been demonstrated in the murine models, experimental autoimmune encephalitis (EAE) and non-obese mouse diabetes (NOD). It is clear that inflammatory Th1 CD4+ T-cells are pivotal in the development of both EAE and NOD [65,66]. Encephalitogenic or diabetegenic Th1 T-cells can be genetically modified to deliver immunosuppressive cytokines which can limit the extent of the disease [67]. For example, delivery of IL-4 by retrovirus-transduced encephalitogenic T-cells delays onset and reduces severity of EAE induced by immunisation against myelin basic protein [68]. Similarly, islet-specific Th1 lymphocytes, transfected to express IL-10, prevent adoptively-transferred diabetes in NOD mice [69]. These results demonstrate that tissue-specific T-cells can be altered genetically to skew the Th1/Th2 environment, ultimately changing the course of the autoimmune disease. The genetic alterations of T-cells specific for cancer would take reverse strategy. Tumour-specific T-cells could be manipulated to increase the Th1 type response at the site of tumour to further enhance inflammation. Target cytokines for overexpression might include IFN-γ, TNF-α and IL-2.

Tumour-specific T-cells can also be genetically engineered to control their *in vivo* growth capabilities. Since many human tumours grow slowly, treatment will likely require a sustained T-cell response to ensure eradication of all micrometastases as well as destruction of the primary tumour. Transferred T-cells, particularly CTL, are extremely short-lived in the absence of supplemental help and stimulation as previously discussed [7]. Aimed at improving the ability to manipulate the *in vivo* growth of tumour-specific T-cells, Evans and colleagues have developed a chimeric GM-CSF/IL-2 receptor which, when transduced into CTL, results in GM-CSF-sensitive proliferation mediated through the IL-2 signalling mechanism [70]. It is envisioned that systemic delivery of GM-CSF could be used to expand only transduced T-cells while avoiding the toxicities associated with IL-2 administration. In addition, the receptor is constitutively expressed and subject to local autocrine activation within the immune microenvironment.

Redirecting T-cell antigen-specificity is also possible using several different methods. One method is to transfect a TCR with known specificity into naive T-cells. As an example, Calogero and colleagues transduced Jurkat T-cells to express an HLA-A2-restricted αβ-TCR gene specific for a MAGE 3 peptide [71]. The resulting T-cell line was activated in response to both T2 cells and a melanoma cell line loaded with MAGE 3 peptide. A limitation of this technique is that in many cancers, TCRs and antigens are not adequately defined. However, in cases where antigens and TCRs are known, this technique, potentially, could be applied to naive non-specific T-cells following inactivation of their endogenous TCR genes.

Another retargeting method is to produce signalling receptors containing an extracellular antigen-specific antibody fused to an intracellular domain that is able to mediate T-cell activation. In one recent study, chimeric receptors containing an extracellular antibody and the Fc receptor γ signalling chain have been made against the colon cancer-associated protein EGP40 [72]. This chimeric receptor, GA733, when transduced into human T-cells conferred both cytokine production and cytolytic activity against EGP40+ colon cancer cells. In another study, Rossig and colleagues recently reported the generation of a T-cell line with dual recognition for EBV antigens and the neuroblastoma ganglioside antigen, GD2 [73]. EBV-specific T-cell lines were transfected with a construct containing the TCR-ζ-chain fused to variable domains of an anti-GD2 antibody. The resulting cells could potentially be maintained *in vivo* and *in vitro* with autologous EBV-infected cells.

In addition to enhancing T-cell function, immunosuppression may need to be circumvented during adoptive T-cell therapy. Infused tumour-specific T-cells, like endogenous tumour-specific T-cells, are likely targets of active systemic immunosuppression. Although multiple mechanisms of immunosuppression have been identified, recent investigations have focused extensively on T-regulatory cells (Tregs) [74-78], which play a key role in the maintenance of immune tolerance to self antigens. Tregs constitute a homogenous population of CD4+ CD25+ T-cells and are selected for in the thymus by self peptides *via* high affinity TCRs [79]. Tregs represent up to 6% of circulating PBMC in humans [75-77]. Selection of Tregs represents an alternative to clonal deletion of T-cells with high affinity TCRs against self antigens. Tregs directly inhibit the growth and function of antigen-specific T-cells by direct cell-to-cell contact [75-77], and recent findings suggest that Tregs proliferate in response to IL-2 in the absence of TCR stimulation. In addition to a direct suppressive effect on T-cells, Tregs have also been shown to down-modulate the co-stimulatory molecules, CD80 and CD86, on APC [80].

Studies in murine cancer models have suggested a role for Tregs in mediating evasion of tumour cells from immune destruction. Onizuka and colleagues demonstrated that rejection of tumour could be enhanced against 6/8 different murine tumours, including leukaemia, myeloma and sarcomas by depleting Tregs[81]. In that study, tumours were rejected in mice that had been previously treated with a single bolus dose of anti-CD25 antibody 4 days earlier. The injections of anti-CD25 antibody resulted in significantly reduced levels of circulating CD25+ T-cells. Similar findings were reported by Shimizu and colleagues who demonstrated that depletion of CD25+ T-cells results in the endogenous generation of antitumour immunity that is directed against a broad spectrum of tumours [82]. These encouraging findings that Tregs may play a key role in suppressing endogenous murine antitumour immunity have led investigators to examine the role of Tregs in mediating evasion of human tumours. Woo and colleagues recently reported evidence for increased levels of Tregs associated with both non-small cell lung cancer (NSCLC) and late-stage ovarian cancer [78]. Compared with autologous peripheral blood T-cells, there was an increased number (approximately 1.5 - 2.0-fold) of CD25+ CD4+ T-cells associated with either the tumour-infiltrating lymphocytes of NSCLC or tumour-associated lymphocytes of ovarian cancer. The CD25+ CD4+ T-cells were isolated and examined for cytokine release and found to secrete an immunosuppressive cytokine, TGF-β. Overcoming the immunosuppressive properties of these cells by direct inhibition or by swamping the effects of Tregs by a variety of mechanisms could be an important objective in designing therapeutically effective adoptive T-cell transfer. In the same study, it was also observed that the ovarian cancer patients, but not the NSCLC patients, had increased levels of CD25+ CD4+ T-cells within the peripheral blood compartment suggesting that strategies may need to be designed to neutralise suppressor activity during *ex vivo* expansion if using PBMC as the source of T-cells.

In addition to Tregs, other cells of haematopoietic origin have been identified that can suppress immunity. Defects in maturation pathways of dendritic cells lead to the accumulation of immature myeloid cells (ImC) in most cancer patients to levels 5 times higher than that observed in normal healthy individuals (< 3%) [83]. ImC, like Tregs, when added to cultures significantly inhibit antigen-specific T-cell proliferation. ImC can be induced to differentiate to mature DC in the

presence of all-*trans*-retinoic acid which simultaneously reduces their ability to inhibit T-cell function. It appears that the accumulation of ImC is a direct result of tumour presence since surgical removal of tumour can at least partially reverse these defects. The identification of suppressive haematopoietic T-cells in cancer patients is important and suggests that adoptive T-cells therapy, as well as tumour antigen-specific *ex vivo* expansion, can be improved through prior depletion of either Tregs or ImC.

6. Applications of imaging to immunotherapy

Recently developed biochemical imaging methods, in particular PET, play an increasing role in clinical oncology [84]. PET imaging may be helpful in the development of adoptive immunotherapy, in particular in three areas:

- monitoring tumour response to treatment
- tracking T-cell homing
- measuring the heterogeneity of antigen expression

The standard approach to measuring tumour response is to look for a decrease in tumour size as tumour cells die and the tumour mass shrinks [85]. However, size changes can lag cellular responses by weeks to months and for cytostatic therapies, tumour size may not change at all. It would therefore be logical to use biochemical imaging to look for evidence of tumour response earlier in the course of treatment. In this regard, PET imaging using $^{[F\text{-}18]}$fluorodeoxyglucose (FDG) has shown that tumour glucose metabolism can decline early in response to successful cytotoxic chemotherapy, well in advance of changes in tumour size [86,87]. For immunotherapy, because a local immune response at the tumour site may be energy requiring, it may be difficult to evaluate tumour response by FDG PET alone. Recent work with PET tracers of cellular proliferation, such as $^{[C\text{-}11]}$thymidine, has shown that measuring tumour cellular proliferation provides an early and quantitative estimate of tumour response to cytotoxic chemotherapy [87,88] and that the early changes in cellular proliferation in response to therapy are larger and more consistent than changes in glucose metabolism [87]. The combination of glucose metabolism and cellular proliferation measurements using PET, over the course of immunotherapy, will provide unique insights into the mechanisms and timing of the immune response *in vivo*.

To date, measuring the response of tumours to adoptive T-cell therapy has been limited to techniques that are restricted in their ability to quantify real time *in vivo* T-cell trafficking and homing. Indirect methods of monitoring therapy such as limiting dilution assays and ELISPOT can underestimate the number of antigen-specific T-cells at the tumour site, while more direct methods such as tetramer complexes are restricted to select MHC molecules. The radiolabelling of T-cells to allow *in vivo* trafficking offers promise in understanding tumour-host interactions, T-cell expansion and T-cell homing during adoptive T-cell therapy. In addition to non-specific labelling of *ex vivo* expanded T-cells, molecular imaging approaches targeting the expression of specific genes using reporters designed to work with particular PET tracers [89] may be able to take advantage of T-cell markers specific for different T-cell subsets with unique functional phenotypes. Imaging the homing of T-cells to tumours and regional lymph nodes can provide new insight into tumour-host interactions. In the setting of adoptive T-cell therapy, these methods can elucidate the functional interaction of different T-cell subsets with tumour cells and other effector arms of the immune system.

One potential explanation for the lack of success with early trials of antigen specific T-cell therapy is the development of tumour antigen-loss variants. Tumours may escape recognition by adoptively transferred T-cells by downregulation of antigens resulting in a tumour that can evade detection and destruction. Measuring the heterogeneity of antigen expression using radiolabelled antibodies may help identify alterations in antigen expression as a cause of therapy failure. This can be done using the single-photon emitting isotope $^{[131]}$I, as in the recently described approach which predicted Herceptin efficacy and cardiotoxicity [90]; or possibly with the positron-emitter, $^{[124]}$I, to provide a more quantitative measure of antigen expression than can be achieved using $^{[131]}$I and conventional radiotracer imaging.

The application of PET imaging technology to measuring tumour metabolism and proliferation, T-cell trafficking and antigen expression in adoptive T-cell therapy may yield insights into the mechanisms underlying functional adoptive immunotherapy and host-tumour interactions. Such studies are currently underway in the authors laboratory and others. Learning more about the functional interactions between T-cells and tumour cells will enable the development of more specific and long lasting responses to tumours and ultimately improve the efficacy of adoptive T-cell therapy.

7. Conclusion

Evidence from both mouse experiments and human clinical trials suggests that the most effectiveT-cell populations will be those that aim to reconstitute full T-cell immunity representing both major T-cell subsets, CTL and Th. In order to generate an adequate T-cell population, *ex vivo*, appropriate culture conditions must be established and may be different for each individual T-cell subset or antigen-presentation system. A number of cytokines have been cloned and produced that can have important effects in culture when used with IL-2. Furthermore, novel methods of antigen-presentation have been established to facilitate *ex vivo* expansion. Recent progress in molecular biology has led to the development of more efficient methods for cloning, altering and transfecting T-cells in order to improve important T-cell characteristics such as antigen-specificity and *in vivo* longevity. Finally, the implementation of *in vivo* imaging using PET should provide a greater understanding of those characteristics that T-cells must possess in order to effectively home to and eradicate tumour.

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Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. CHEEVER MA, CHEN W: Therapy with cultured T cells: principles revisited. *Immunol. Rev.* (1997) 157:177-194.

•• **A well-done overview of methods for *ex vivo* expansion and *in vivo* maintenance of tumour antigen-specific T-cells in a murine leukaemia model.**

2. ROSKROW MA, SUZUKI N, GAN Y *et al.*: Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes for the treatment of patients with EBV-positive relapsed Hodgkin's disease. *Blood* (1998) 91:2925-2934.

3. ROONEY CM, SMITH CA, NG CY *et al.*: Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet* (1995) 345:9-13.

4. HESLOP HE, NG CY, LI C *et al.*: Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nat. Med.* (1996) 2:551-555.

5. SPRENT J, KISHIMOTO H: The thymus and central tolerance. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* (2001) 356:609-616.

6. LECHLER R, CHAI JG, MARELLI-BERG F, LOMBARDI G: T-cell anergy and peripheral T-cell tolerance. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* (2001) 356:625-637.

7. DUDLEY ME, WUNDERLICH J, NISHIMURA MI *et al.*: Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. *J. Immunother.* (2001) 24:363-373.

8. ROSENBERG SA, LOTZE MT, MUUL LM *et al.*: A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N. Engl. J. Med.* (1987) 316:889-897.

9. FISHER RI, COLTMAN CAJ, DOROSHOW JH *et al.*: Metastatic renal cancer treated with interleukin-2 and lymphokine- activated killer cells. A Phase II clinical trial. *Ann. Intern. Med.* (1988) 108:518-523.

10. STEWART JA, BELINSON JL, MOORE AL *et al.*: Phase I trial of intraperitoneal recombinant interleukin-2/lymphokine-activated killer cells in patients with ovarian cancer. *Cancer Res.* (1990) 50:6302-6310.

11. LUM LG: T cell-based immunotherapy for cancer: a virtual reality? *CA Cancer J. Clin.* (1999) 49:74-100, 65.

12. BELLDEGRUN A, MUUL LM, ROSENBERG SA: Interleukin 2 expanded tumor-infiltrating lymphocytes in human renal cell cancer: isolation, characterization, and antitumor activity. *Cancer Res.* (1988) 48:206-214.

13. RATTO GB, ZINO P, MIRABELLI S *et al.*: A randomized trial of adoptive immunotherapy with tumor-infiltrating lymphocytes and interleukin-2 *versus* standard therapy in the postoperative treatment of resected nonsmall cell lung carcinoma. *Cancer* (1996) 78:244-251.

14. ROSENBERG SA, YANNELLI JR, YANG JC *et al.*: Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J. Natl. Cancer Inst.* (1994) 86:1159-1166.

15. SCHWARTZENTRUBER DJ, HOM SS, DADMARZ R *et al.*: *In vitro* predictors of therapeutic response in melanoma patients receiving tumor-infiltrating lymphocytes and interleukin-2. *J. Clin. Oncol.* (1994) 12:1475-1483.

16. TO WC, WOOD BG, KRAUSS JC *et al.*: Systemic adoptive T-cell immunotherapy in recurrent and metastatic carcinoma of the head and neck: a Phase 1 study. *Arch. Otolaryngol. Head Neck Surg.* (2000) 126:1225-1231.

17. PLAUTZ GE, MILLER DW, BARNETT GH *et al.*: T cell adoptive immunotherapy of newly diagnosed gliomas. *Clin. Cancer Res.* (2000) 6:2209-2218.

18. ROONEY CM, ROSKROW MA, SUZUKI N, NG CY, BRENNER MK, HESLOP H: Treatment of relapsed Hodgkin's disease using EBV-specific cytotoxic T cells. *Ann. Oncol.* (1998) 9(Suppl. 5):S129-S132.

19. WALTER EA, GREENBERG PD, GILBERT MJ *et al.*: Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N. Engl. J. Med.* (1995) 333:1038-1044.

20. DISIS ML, KNUTSON KL, SCHIFFMAN K, RINN K, MCNEEL DG: Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. *Breast Cancer Res. Treat.* (2000) 62:245-252.

21. KNUTSON KL, SCHIFFMAN K, DISIS ML: Immunization with a HER-2/neu helper peptide vaccine generates of HER-2/neu CD8 T cell immunity in cancer patients. *J. Clin. Invest.* (2001) 107:477-484.

•• **Demonstrates that late stage cancer patients with HER-2/neu positive tumours generate HER-2/neu-specific CD8 T-cell immunity following immunisation with a Th cell peptide vaccine of which each peptide contained nested HLA-A2 binding motifs. The resulting CD8 T-cells persisted for up to one year.**

22. KNUTSON KL, CROSBY P, DISIS ML: Isolation of HER-2/neu (HER2) specific T cell clones from a cancer patient following immunization with a HER2 peptide vaccine. *Proc. Am. Assoc. Cancer Res.* (2000) 40:310-310. Abstract.

23. KNUTSON KL, DISIS ML: Clonal diversity of the T cell repertoire responding to a dominant HLA-A2 epitope of HER-2/neu after active immunzation. *(Submitted)* (2001).

24. REDDISH M, MACLEAN GD KOGANTY RR *et al.*: Anti-MUC1 class I restricted CTLs in metastatic breast cancer patients immunized with a synthetic MUC1 peptide. *Int. J. Cancer* (1998) 76:817-823.

25. MATLOUBIAN M, CONCEPCION RJ, AHMED R: CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J. Virol.* (1994) 68:8056-8063.

26. ANDREASEN SO, CHRISTENSEN JE, MARKER O, THOMSEN AR: Role of CD40 ligand and CD28 in induction and maintenance of antiviral CD8+ effector T cell responses. *J. Immunol.* (2000) **164**:3689-3697.
27. BATTEGAY M, MOSKOPHIDIS D, RAHEMTULLA A, HENGARTNER H, MAK TW, ZINKERNAGEL RM: Enhanced establishment of a virus carrier state in adult CD4+ T-cell-deficient mice. *J. Virol.* (1994) **68**:4700-4704.
28. THOMSEN AR, JOHANSEN J, MARKER O, CHRISTENSEN JP: Exhaustion of CTL memory and recrudescence of viremia in lymphocytic choriomeningitis virus-infected MHC class II-deficient mice and B cell-deficient mice. *J. Immunol.* (1996) **157**:3074-3080.
29. COHEN PA, PENG L, PLAUTZ GE, KIM JA, WENG DE, SHU S: CD4+ T cells in adoptive immunotherapy and the indirect mechanism of tumor rejection. *Crit. Rev. Immunol.* (2000) **20**:17-56.
30. KAYAGAKI N, YAMAGUCHI N, NAKAYAMA M *et al.*: Involvement of TNF-related apoptosis-inducing ligand in human CD4+ T cell-mediated cytotoxicity. *J. Immunol.* (1999) **162**:2639-2647.
31. ABE K, KURAKIN A, MOHSENI-MAYBODI M, KAY B, KHOSRAVI-FAR R: The complexity of TNF-related apoptosis-inducing ligand. *Ann. NY Acad. Sci.* (2000) **926**:52-63.
32. DISIS ML, CHEEVER MA: HER-2/neu oncogenic protein: issues in vaccine development. *Crit. Rev. Immunol.* (1998) **18**:37-45.
33. DISIS ML, GRABSTEIN KH, SLEATH PR, CHEEVER MA: Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. *Clin. Cancer Res.* (1999) **5**:1289-1297.
34. GATELY MK, WILSON DE, WONG HL: Synergy between recombinant interleukin 2 (rIL 2) and IL 2-depleted lymphokine-containing supernatants in facilitating allogeneic human cytolytic T lymphocyte responses *in vitro*. *J. Immunol.* (1986) **136**:1274-1282.
35. WOLF SF, TEMPLE PA, KOBAYASHI M *et al.*: Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J. Immunol.* (1991) **146**:3074-3081.
36. GATELY MK, DESAI BB, WOLITZKY AG *et al.*: Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). *J. Immunol.* (1991) **147**:874-882.
37. UHEROVA P, CONNICK E, MAWHINNEY S, SCHLICHTEMEIER R, SCHOOLEY RT, KURITZKES DR: *In vitro* effect of interleukin-12 on antigen-specific lymphocyte proliferative responses from persons infected with human immunodeficiency virus Type 1. *J. Infect. Dis.* (1996) **174**:483-489.
38. MCFARLAND EJ, HARDING PA, MAWHINNEY S, SCHOOLEY RT, KURITZKES DR: *In vitro* effects of IL-12 on HIV-1-specific CTL lines from HIV-1-infected children. *J. Immunol.* (1998) **161**:513-519.
39. TRINCHIERI G: Function and clinical use of interleukin-12. *Curr. Opin. Hematol.* (1997) **4**:59-66.
40. DISIS ML, GOOLEY TA, RINN K *et al.*: Generation of T Cell immunity to the HER-2/neu protein after active immunization with HER-2/neu peptide-based vaccines. *J. Clin. Oncol. (in press)* (2001).
41. KNUTSON KL, DISIS ML: IL-12 enhances the function and number of tumor antigen-specific Th1 lymphocytes during *ex vivo* expansion. *(Submitted)* (2001).
42. TAN JT, DUDL E, LEROY E *et al.*: IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc. Natl. Acad. Sci. USA* (2001) **98**:8732-8737.
43. SCHULTZE JL, SEAMON MJ, MICHALAK S, GRIBBEN JG, NADLER LM: Autologous tumor infiltrating T cells cytotoxic for follicular lymphoma cells can be expanded *in vitro*. *Blood* (1997) **89**:3806-3816.
44. TSAI V, KAWASHIMA I, KEOGH E, DALY K, SETTE A, CELIS E: *In vitro* immunization and expansion of antigen-specific cytotoxic T lymphocytes for adoptive immunotherapy using peptide-pulsed dendritic cells. *Crit. Rev. Immunol.* (1998) **18**:65-75.
45. GRABSTEIN KH, EISENMAN J, SHANEBECK K *et al.*: Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *Science* (1994) **264**:965-968.
46. MA A, BOONE DL, LODOLCE JP: The pleiotropic functions of interleukin 15: not so interleukin 2-like after all. *J. Exp. Med.*. (2000) **191**:753-756.
47. WALDMANN TA, DUBOIS S, TAGAYA Y: Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy. *Immunity* (2001) **14**:105-110.
48. WENINGER W, CROWLEY MA, MANJUNATH N, VON ANDRIAN UH: Migratory properties of naive, effector, and memory CD8(+) T cells. *J. Exp. Med.* (2001) **194**:953-966.
49. DYBUL M, MERCIER G, BELSON M *et al.*: CD40 ligand trimer and IL-12 enhance peripheral blood mononuclear cells and CD4+ T cell proliferation and production of IFN-gamma in response to p24 antigen in HIV-infected individuals: potential contribution of anergy to HIV-specific unresponsiveness. *J. Immunol.* (2000) **165**:1685-1691.
50. GREWAL IS, FLAVELL RA: CD40 and CD154 in cell-mediated immunity. *Ann. Rev. Immunol.* (1998) **16**:111-135.
51. KEDL RM, JORDAN M, POTTER T, KAPPLER J, MARRACK P, DOW S: CD40 stimulation accelerates deletion of tumor-specific CD8(+) T cells in the absence of tumor-antigen vaccination. *Proc. Natl. Acad. Sci. USA* (2001) **98**:10811-10816.
52. TERHEYDEN P, STRATEN P, BROCKER EB, KAMPGEN E, BECKER JC: CD40-ligated dendritic cells effectively expand melanoma-specific CD8+ CTLs and CD4+ IFN-gamma-producing T cells from tumor-infiltrating lymphocytes. *J. Immunol.* (2000) **164**:6633-6639.
53. REILLY RT, MACHIELS JP, EMENS LA *et al.*: The collaboration of both humoral and cellular HER-2/neu-targeted immune responses is required for the complete eradication of HER-2/neu-expressing tumors. *Cancer Res.* (2000) **61**:880-883.
54. BANCHEREAU J, STEINMAN RM: Dendritic cells and the control of immunity. *Nature* (1998) **392**:245-252.
55. KUROKAWA T, OELKE M, MACKENSEN A: Induction and clonal expansion of tumor-specific cytotoxic T lymphocytes from renal cell carcinoma patients after stimulation with autologous dendritic cells loaded with tumor cells. *Int. J. Cancer* (2001) **91**:749-756.
56. YEE C, SAVAGE PA, LEE PP, DAVIS MM, GREENBERG PD: Isolation of high avidity melanoma-reactive CTL from

heterogeneous populations using peptide-MHC tetramers. *J. Immunol.* (1999) 162:2227-2234.

• **Describes the utility of using tetramers to isolate high avidity, antigen-specific peptide-specific T cells for ex vivo clonal expansion. Discusses the application to adoptive T cell therapy of melanoma.**

57. HEISER A, MAURICE MA, YANCEY DR *et al.*: Induction of polyclonal prostate cancer-specific CTL using dendritic cells transfected with amplified tumor RNA. *J Immunol.* (2000) 166:2953-2960.

58. HEISER A, MAURICE MA, YANCEY DR, COLEMAN DM, DAHM P, VIEWEG J: Human dendritic cells transfected with renal tumor RNA stimulate polyclonal T-cell responses against antigens expressed by primary and metastatic tumors. *Cancer Res.* (2001) 61:3388-3393.

59. MEYER ZB, METZGER J, HERMANN C, NICKLISCH N, PESCHEL C, BERNHARD H: The generation of both T killer and Th cell clones specific for the tumor-associated antigen her2 using retrovirally transduced dendritic cells. *J. Immunol.* (2001) 167:1712-1719.

•• **Demonstrates that HER-2/neu-specific T-cells, both helper and CTL, can be expanded selectively in culture using autologous HER-2/neu-expressing DC derived from CD34 progenitor cells. Discusses potential application to adoptive T-cell immunotherapy and the generation of high affinity antigen-specific T-cells.**

60. KE Y, MA H, KAPP JA: Antigen is required for the activation of effector activities, whereas interleukin 2 Is required for the maintenance of memory in ovalbumin-specific, CD8+ cytotoxic T lymphocytes. *J. Exp. Med.* (1998) 187:49-57.

61. MORRIS A, VETTO JT, RAMSTAD T *et al.*: Induction of anti-mammary cancer immunity by engaging the OX-40 receptor *in vivo*. *Breast Cancer Res. Treat.* (2001) 67:71-80.

62. WEINBERG AD, RIVERA MM, PRELL R *et al.*: Engagement of the OX-40 receptor *in vivo* enhances antitumor immunity. *J. Immunol.* (2000) 164:2160-2169.

63. KJAERGAARD A, WEINBERG A, SHU S: Characteristics of antibody-mediated OX-40R ligation in augmentation of adoptive T-cell immunotherapy of advanced tumor. *Proc. Am. Assn. Canc Res.* (2001) 42:328. Abstract.

64. GREENBERG PD, FINCH RJ, GAVIN MA *et al.*: Genetic modification of T-cell clones for therapy of human viral and malignant diseases. *Cancer J. Sci. Am.* (1998) 4 (Suppl. 1):S100-S105.

• **Underscores the potential application and importance of genetically modifying T-cells for improving function and extending life.**

65. STEINMAN L: Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. *Cell* (1996) 85:299-302.

66. TISCH R, MCDEVITT H: Insulin-dependent diabetes mellitus. *Cell* (1996) 85:291-297.

67. TUOHY VK, MATHISEN PM: T-cell design: optimizing the therapeutic potential of autoreactive T cells by genetic modification. *Res. Immunol.* (1998) 149:834-842.

•• **The autoreactive T-cells of diabetes and EAE can be genetically altered to deliver immunosuppressive molecules to sites of inflammation in order to suppress disease symptoms.**

68. SHAW MK, LORENS JB, DHAWAN A *et al.*: Local delivery of interleukin 4 by retrovirus-transduced T lymphocytes ameliorates experimental autoimmune encephalomyelitis. *J. Exp. Med.* (1997) 185:1711-1714.

69. MORITANI M, YOSHIMOTO K, II S *et al.*: Prevention of adoptively transferred diabetes in nonobese diabetic mice with IL-10-transduced islet-specific Th1 lymphocytes. A gene therapy model for autoimmune diabetes. *J. Clin. Invest.* (1996) 98:1851-1859.

70. EVANS LS, WITTE PR. FELDHAUS AL *et al.*: Expression of chimeric granulocyte-macrophage colony-stimulating factor/ interleukin 2 receptors in human cytotoxic T lymphocyte clones results in granulocyte-macrophage colony-stimulating factor-dependent growth. *Hum. Gene Ther.* (1999) 10:1941-1951.

71. CALOGERO A, HOSPERS GA, KRUSE KM *et al.*: Retargeting of a T cell line by anti MAGE-3/HLA-A2 alpha beta TCR gene transfer. *Anti-Cancer Res.* (2000) 20:1793-1799.

72. DALY T, ROYAL RE, KERSHAW MH *et al.*: Recognition of human colon cancer by T cells transduced with a chimeric receptor gene. *Cancer Gene Ther.* (2000) 7:284-291.

73. ROSSIG C, NUCHTERN JG, ROONEY CM, BRENNER MK: Targeting of tumor cells with EBV-specific human T lymphocytes transducred to express tumor-specific chimeric T cell receptor genes. *Proc. Am. Assoc. Cancer Res.* (2001) 42:845. Abstract.

74. JONULEIT H, SCHMITT E, STASSEN M, TUETTENBERG A, KNOP J, ENK AH: Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J. Exp. Med.* (2001) 193:1285-1294.

75. LEVINGS MK, SANGREGORIO R, RONCAROLO MG: Human CD25(+)CD4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded *in vitro* without loss of function. *J. Exp. Med.* (2001) 193:1295-1302.

76. DIECKMANN D, PLOTTNER H, BERCHTOLD S, BERGER T, SCHULER G: *Ex vivo* isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J. Exp. Med.* (2001) 193:1303-1310.

77. STEPHENS LA, MOTTET C, MASON D, POWRIE F: Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity *in vitro*. *Eur. J. Immunol.* (2001) 31:1247-1254.

78. WOO EY, CHU CS, GOLETZ TJ *et al.*: Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res.* (2001) 61:4766-4772.

79. JORDAN MS, BOESTEANU A, REED AJ *et al.*: Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* (2001) 2:301-306.

80. CEDERBOM L, HALL H, IVARS F: CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur. J. Immunol.* (2000) 30:1538-1543.

81. ONIZUKA S, TAWARA I, SHIMIZU J, SAKAGUCHI S, FUJITA T, NAKAYAMA E: Tumor rejection by *in vivo* administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res.* (1999) 59:3128-3133.

82. SHIMIZU J, YAMAZAKI S, SAKAGUCHI S: Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J. Immunol.* (1999) 163:5211-5218.

83. ALMAND B, CLARK JI, NIKITINA E *et al.*: Increased production of immature

myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J. Immunol.* (2001) 166:678-689.

84. EARY JF: Nuclear medicine in cancer diagnosis. *Lancet* (1999) 354:853-857.
85. HUSBAND JE: Monitoring tumour response. *Eur. Radiol.* (1996) 6:775-785.
86. WAHL RL, QUINT LE CIESLAK RD, AISEN AM, KOEPPE RA, MEYER CR: 'Anatometabolic' tumor imaging: fusion of FDG PET with CT or MRI to localize foci of increased activity. *J. Nucl. Med.* (1993) 34:1190-1197.
87. SHIELDS AF, MANKOFF DA, LINK JM *et al.*: Carbon-11-thymidine and FDG to measure therapy response. *J. Nucl. Med.* (1998) 39:1757-1762.
88. MANKOFF DA, DEHDASHTI F, SHIELDS AF: Characterizing tumors using metabolic imaging: PET imaging of cellular proliferation and steroid receptors. *Neoplasia* (2000) 2:71-88.
89. GAMBHIR SS, HERSCHMAN HR, CHERRY SR *et al.*: Imaging transgene expression with radionuclide imaging technologies. *Neoplasia* (2000) 2:118-138.
90. BEHR TM, BEHE M, WORMANN B: Trastuzumab and breast cancer. *N. Engl. J. Med.* (2001) 345:995-996.

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